Universidade de Aveiro Departamento de Química
Ano 2014

Rui Pedro Fonseca Ferreira da Silva

Extração supercrítica de *Eichhornia crassipes*

Supercritical fluid extraction of *Eichhornia crassipes*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica do Professor Doutor Carlos Manuel Silva, Professor Auxiliar do Departamento de Química da Universidade de Aveiro, e coorientação científica do Mestre Marcelo Morais Rodrigues de Melo, bolseiro de investigação do Departamento de Química da Universidade de Aveiro.

Aos meus pais
À Belinda

o júri
presidente

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Professora Auxiliar do Departamento de Química da Universidade de Aveiro

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palavras-chave

Jacinto de água, *Eichhornia crassipes*, fitoesteróis, estigmasterol, extração supercrítica, GC-MS, fenólicos totais, atividade antioxidante, curva de extração, rendimento de extração, modelação, valorização.

resumo

Nesta dissertação pretendeu-se avaliar o potencial da *Eichhornia crassipes* colhida na Pateira de Fermentelos, Águeda, como uma possível fonte de fitoesteróis, nomeadamente estigmasterol, extraídos com recurso a Soxhlet com diclorometano e usando dióxido de carbono supercrítico. A extração supercrítica surge nesta tese como alternativa a extrações convencionais com solventes orgânicos, pretendendo-se avaliar o seu potencial em termos de capacidade e seletividade para o estigmasterol. Em ambos os casos, os extratos obtidos foram caracterizados por diferentes técnicas: GC-MS, quantificação de fenólicos totais e atividade antioxidante.

Com este objetivo, foi elaborado um plano experimental de trabalho. Primeiramente, a planta colhida, separada nas suas diferentes partes morfológicas (raiz, caule, folha e flor), seca e triturada. Em segundo lugar, as extrações Soxhlet foram efetuadas e os extratos analisados por GC-MS. Estas extrações revelaram uma concentração elevada de estigmasterol nos caules e folhas (15.0% e 13.5%; mg/100mg$_{extract}$) e um rendimento total de 1.12, 2.65, 3.30 e 4.45 wt.% para raízes, caules, folhas e flores, respectivamente. De seguida, foram realizadas extrações sólido-líquido com misturas metanol:água e os extratos analisados relativamente à composição em fenólicos totais e atividade antioxidante. Estes resultados evidenciaram que, de todas as partes morfológicas da planta, as folhas são as que possuem uma maior quantidade de fenólicos totais assim como maior atividade antioxidante. Por fim, foram conduzidas várias extrações supercríticas. Começou-se por alguns ensaios preliminares, usando uma mistura de todas as partes da planta, que mostraram um baixo rendimento total, mas uma elevada concentração em estigmasterol (31 mg/100mg$_{extract}$). A segunda etapa de extrações supercríticas visou a medição de curvas de extração, usando as partes morfológicas da biomassa que apresentavam maior concentração de estigmasterol, ou seja, caules e folhas. Estas extrações revelaram que a influência da temperatura é praticamente desprezável, que o tempo ótimo de extração é de cerca de 1 h, mas que a concentração máxima de estigmasterol se obtém ao fim de duas horas. Em termos de modelação das curvas cumulativas de extração, mostrou-se que o processo supercrítico é predominantemente controlado por difusão interna.

Em conclusão, os resultados obtidos demonstraram que a extração supercrítica de *E. crassipes* pode ser considerada uma tecnologia promissora para a produção de extratos enriquecidos em estigmasterol, podendo desta forma viabilizar uma implementação comercial.

keywords

Water hyacinth, *Eichhornia crassipes*, phytosterols, stigmasterol, supercritical fluid extraction, GC-MS, total phenolics, antioxidant activity, modeling, valorization.

abstract

The aim of this thesis was to evaluate the potential of *Eichhorina crassipes* harvested from Pateira de Fermentelos, Águeda, as a source of phytosterols, namely stigmasterol, by Soxhlet and supercritical fluid extraction. Supercritical extractions were used in this thesis as an alternative to conventional techniques that use organic solvents, allowing the assessment of both extractions in terms of concentration, yield and selectivity for stigmasterol. In both cases chemical evaluation was carried out using different techniques: GC-MS, total phenol content and antioxidant activity.

With this intent, an experimental work plan was established. First, the biomass was harvested and divided in its different morphological parts, roots, stalks, leaves and flowers. Secondly, soxhlet extractions with dichloromethane were performed and the extracts analyzed by GC-MS. This step revealed a strong stigmasterol concentration in stalks and leaves (15.0% and 13.5%; $mg/100mg_{extract}$) and a total yield value of 1.12, 2.65, 3.30 and 4.45 wt.% respectively. Thirdly, solid liquid extractions were performed and total phenolic and antioxidant activity were assessed, revealing that leaves possessed higher phenolic content and antioxidant activity than all other morphological parts.
Finally, supercritical fluid extractions (SFE) were performed. These were divided in two stages. In the preliminary stage, a mixture of all parts where submitted to extraction revealing a lower overall extraction yield but higher stigmasterol concentration (31% $mg/100mg_{extract}$).
The second phase of SFE was performed in order to obtain the overall extraction curves (OEC). These were performed with a biomass mixture that revealed a higher stigmasterol content, namely stalks and leaves. These extractions demonstrated that temperature had little effect on the total yield, that the optimal time of extraction was of one hour and the maximum stigmasterol concentration was achieved after two hours of extraction. Regarding the modeling of the extraction curves, internal diffusion revealed to be the dominant mass transfer mechanism.

In sum, these results show that supercritical extraction of *E. crassipes* biomass can become a promising extraction technology, generating rich stigmasterol extracts, opening a possible window for its commercial implementation.

INDEX

NOMENCLATURE

b	Logistic model parameter
D_{AB}	Diffusivity
D_m	Solute diffusivity in the solid
d_p	Particle diameter
h	Height position
H	Bed height
IC_{50}	Inhibition concentration value
k_d	Desorption constant
k_f	Convective mass transfer coefficient
m	mass
P	Pressure
P_c	Critical pressure
Q_{CO2}	Solvent flow rate
R_p	Particle radius
SD	Standard deviation
t	Time
T	Temperature
X_0	Initial solute concentration in the solid

Greek symbols

μ	Viscosity
ρ	Density
σ	Solubility

Abbreviations

AAE	Ascorbic acid equivalent
AAI	Activity antioxidant index
CER	Constant extraction rate
DC	Diffusion-controlled
DFM	Diffusional model
DSM	Desorption model

EFSA	European food safety authority
EU	European Union
F	Flowers
FER	Falling extraction rate
FUSE	Focused ultrasound extraction
GAE	Gallic acid equivalent
GC-MS	Gas chromatography – Mass spectrometry
GRAS	General recognized as safe
HPLC	High-performance liquid chromatography
IS	Internal standard
L	Leaves
LER	Low extraction rate
LM	Logistic model
OEC	Overall extraction curves
R	Roots
R_T	Retention time
S	Stalks
SC-CO_2	Super critical CO_2
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SSPM	Simple Single Plate model
TMS	Trimethylsilyl
TPC	Total phenolic content
TR	Traces
USFE	Ultrasound-assisted supercritical fluid extraction

FIGURES INDEX

TABLES INDEX

SCOPE AND STRUCTURE

Since ancient times, mankind has applied herbs and their derivatives as therapeutic medicines. Herbal medicine, also known as botanical medicine or phytomedicine, refers to the use of a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. People have been applying herbal medicine for treatment, control and management on a vast variety of diseases. It is an evolving practice recorded in both folklore and books of early practitioners. At present, despite the abundance and advance of synthetic drugs, a significant proportion of developing countries' population still depend on traditional medicines to attend to their health care needs [1,2].

According to World Health Organization although the use of herbal extracts in developed countries continued to decrease in the late nineteenth and early twentieth century in some Asian and African countries, 80% of the population depend on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine, making herbal medicines the most lucrative form of traditional medicine, generating billions of euros in revenue [1–3].

Eichhornia crassipes is a fast growing plant native of the Amazon basin that has spread all over the world. This species grows extremely fast causing enormous ecological problems. Pateira de Fermentelos lagoon, located in Águeda, Aveiro, Portugal, faces this problem since the late 90's when this plant was first brought to this location. Since then, great endeavors have taken place in order to control and ultimately eradicate this nonnative plant from this habitat. Mechanical harvest has been by far the most efficient form of control of this species until the present day. In 2007, Águeda's council spent 37506€ and a total of 15540m^3 of this plant biomass was removed [4].

Scientific studies on this species have revealed the potential of this plant as a sorbent for heavy metals from polluted waters and also shown the possibility of this plant extracts being used as a health care solution, but no research was found regarding its chemical characterization. The work presented in this thesis has as one of its main objectives the chemical characterization of *Eichhornia crassipes* harvested from Pateira de Fermentelos basin by both conventional extraction procedures and supercritical fluid extraction, giving a freshly new approach to the local and worldwide problem.

In order to achieve this goal, the following objectives were drawn:

- Evaluation of the potential of *Eichhornia crassipes* as a phytosterols source:
    - Characterization of the lipophilic fraction obtained by Soxhlet with dichloromethane solvent using GC-MS analysis;

    - Characterization of the polar (hydrophilic) fraction obtained by solid-liquid extraction (maceration) with polar solvent (antioxidant activity and total phenolic content);
- Comparison of the extracts obtained by Soxhlet with those obtained by supercritical fluid extraction (SFE) with CO_2;
- Optimization of the SFE operating conditions in order to maximize the stigmasterol yield and concentration;
- Mathematical modeling of the SFE kinetic curves.

The present work is composed by four chapters. The first chapter presents a brief bibliographic revision about the *Eichhornia crassipes* species, its origin and world spread, the nefarious impact on Pateira de Fermentelos and its potential as a phytochemical and medicinal source. Moreover, notions of phytosterols, specifically, their chemical composition, function, sources and applications are also addressed. Finally a brief description of the applied extraction techniques is discussed. The experimental work is presented in the second chapter, where the extractions methodology and characterization techniques are described. The obtained results and their discussion are presented in the third chapter and, finally, the fourth chapter summarizes the main conclusions and suggestions for future work.

1. INTRODUCTION

1.1 WATER HYACINTH *(Eichhornia crassipes)*

1.1.1 ORIGIN, ECOSYSTEM AND WORLD SPREAD

The water hyacinth, *Eichhornia crassipes* (Figure 1), is an invasive plant native of the Amazon basin of Brazil [4–7], whose natural distribution prior to 1800 is not thought to have extended beyond South America. This plant is listed as one of the most productive plants on earth and is considered as the world's worst aquatic plant. Its habitat ranges from tropical desert to subtropical or warm temperate desert to rainforest zones. Optimum temperature for *E. crassipes* growth is 25-30 °C and its growth ceases when water temperature rises above 40°C or falls below 10°C. However, short periods at freezing temperature are tolerated. Optimum pH value varies between 6 and 8, and pH values below 4.5 or above 10 can be damaging. Calcium concentration is important, with an observed threshold of 5 mg/L, below which growth ceases. The plant is also euryhaline, enduring only low levels of salinity making the problem in coastal lagoons dependent on the growth of the weed in the fresh water of the rivers that flow into them. The estimated annual biomass production of water hyacinth varies from 90 to 140 ton of dry matter per hectare depending on geographical and climate factors [6,8–10].

Figure 1 – Visual aspect of the *Eichhornia crassipes* species.

Figure 2 – World map distribution/spread of water hyacinths (adapted from [8]).

Due to its striking flowers, it was deliberately introduced into botanic gardens in many other countries (Figure 2), from which it inevitably spread as a weed [8,10,11]. In Europe, it was possibly introduced as an ornament in the first third of the 20th century, and the first reference to its presence in Portugal dates back to the year 1939. Since then, it has spread over the central-west part of the country through irrigation canals and currently exists in Douro Litoral, Beira Litoral, Estremadura, Ribatejo and Alto Alentejo [4,6,10–12].

Wherever it encounters suitable environmental conditions it can spread with phenomenal rapidity to form vast monotypic stands in lakes, rivers and rice paddy fields obstructing navigation, fishing, and irrigation causing blockage of drainage systems. Its impact on water quality and on the biological diversity is devastating. Water hyacinth was found resistant to chemical, physical, biological or hybrid treatments, and often only an integrated management strategy, biological control, and mechanical harvest can provide a long-term solution to this pest [6,10].

1.1.2 PATEIRA DE FERMENTELOS

Pateira de Fermentelos (Figure 3) is the largest Portuguese freshwater lagoon, it is located in the municipal triangle of Águeda, Aveiro and Oliveira do Bairro, before the confluence of rivers Cértimo and Águeda. Located in the Vouga river basin, this wetland and its surrounding area creates one of the biggest freshwater lagoons of the Iberian Peninsula with an area of 5.3 km^2 and an average depth of 2 meters which supports a

diverse and important aquatic system [4]. This lagoon is threatened by the rapid spread of water hyacinths that was first introduced in the late 90's covering the lagoon canals and water mirror. This large spread has a major impact on the lagoon hydrology, degradation of water quality and depletion of biological activity [4,8,13].

Mechanical harvest was the adopted management practice in the cleaning procedure of the lagoon. In 2007 a total of 15540 m^3 were removed with a total cost of 37506 €, the following year a total of 1992 m^3 were removed with a total cost of 8890 €. The ultimate ending of the harvested biomass is its deposition in an open-pit waterproofed quarry, which will in the future be covered with soil and reforested with autochthonous species [4].

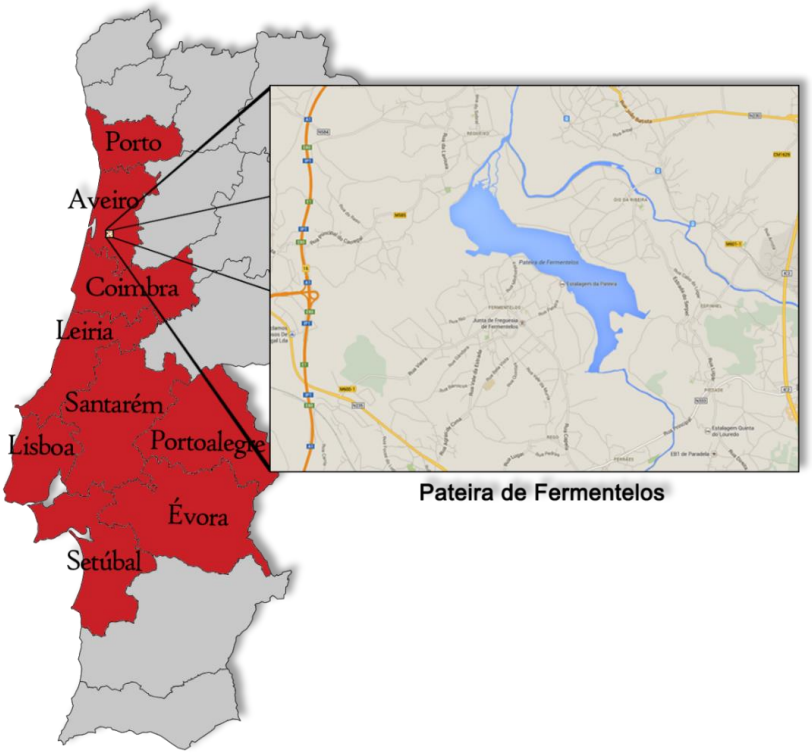


Figure 3 – Spread of *Eichhornia crassipes* in Portugal with focus on the Pateira de Fermentelos location (adapted from [7] and Google Maps).

Nowadays, traditional practices, sports and touristic activities have been restored and protective measures were implemented in order to achieve a sustainable development of this important wetland. Despite constant cleaning, water hyacinths still endure in Pateira de Fermentelos lagoon, and constant surveillance and harvest is needed in order to have control over this fast growing plant [4,7].

Besides being controlled, a deeper knowledge of this species chemical composition could convey in new forms of valorization, along with a sustained and possible profitable management of this fast spreading water plant.

1.1.3 SCIENTIFIC STUDIES

In recent years, different approaches have emerged in order to deal with this invasive and fast growing plant. Because of the rich diversity of this species, it is expected that screening and scientific evaluation of its extracts may prove beneficial for humankind. Scientific studies indicate that it can be used as an organic fertilizer [13,14], in the control of root knot nematodes [15] or employed as an economically cheap biosorbent of heavy metals from aqueous solutions [16]. However, qualitative analysis with different extraction solvents from different plant parts have revealed the presence of various phytochemical compounds like tannins, steroids, terpenoids, alkaloids, flavonoids, quinones, antraquinones and cardiac glycosides [17,18]. Antimicrobial activity, antitumor activity on melanoma, anti-inflammatory activity and anti-oxidative studies have shown that despite being considered the world's worst aquatic plant, it can provide a medical contribution that is yet to be exploited worldwide [19–27].

1.2 HERBS PHYTOCHEMICALS

Herbs have vast ability to synthesize aromatic materials mainly secondary metabolites. Herbs and herbs-based therapies are the source of various modern pharmaceuticals. In many cases, these herbal materials serve as defensive molecules against microorganisms, insects, other plants and herbivores [1,3,28,29].

Herbs extracts have great potential as biologically active compounds against pathogens, including microorganisms [28,29]. The synergetic effect from the association of antibiotics in plant extracts against resistant bacteria leads to new opportunities for the treatment of infectious diseases which empowers the plant extracts as a potential candidate for the development of new drugs. New active compounds have been discovered from a multiplicity of plants species based on the study of traditional medicine [3,26,30,31].

The medicinal and pharmacological actions of medicinal herbs are often dependent on the presence of bioactive compounds called secondary herbal metabolites [1,29,32]. Unlike the ubiquitous macromolecules of primary metabolism (e.g. monosaccharaides, polysaccharides, amino acids, proteins and lipids) which are present in all plants, secondary metabolites with medicinal properties are found only in a few species of plants [3].

Secondary herbal metabolites with reported medicinal properties consist of waxes, fatty acids, alkaloids, terpenoids, phenolics (simple phenolics and flavonoids), glycosides and

phytosterols [1,33,34]. This thesis will focus on phytosterols, mainly stigmasterol, which was the most abundant compound found in the water hyacinth lipophilic extracts.

1.2.1 PHYTOSTEROLS

1.2.1.1 CHEMICAL COMPOSITION

Phytosterols or plant sterols are part of a vast family of isoprenoids showing a fascinating chemical complexity (more than 250 different sterols compounds have been indexed in various plant and marine life) and remarkable diversity of function in living organisms. Plant sterols are steroid alcohols and they resemble cholesterol, the predominant sterol found in animals, both in their chemical structure and their biological function.

Sterols are structurally related to cholesterol but differ from this molecule in the structure of the side chain. They consist of a steroid skeleton with a hydroxyl group attached to the C-3 atom of the A-ring and an aliphatic side chain attached to the C-17 atom of the D-ring. Sterols have a double bond, typically between C-5 and C-6 of the sterol moiety, whereas this bond is saturated in phytostanols [35].

The most common phytosterols are shown in Figure 4: sitosterol (3β-stigmast-5-en-3ol; CAS Number 83-46-5), campesterol (3β-Ergost-5-en-3-ol; CAS Number 474-62-4) and stigmasterol (3β-stigmasta-5,22-dien-3-ol; CAS Number 83-48-7) [35].

1.2.1.1 FUNCTION

Sterols are membrane components and as such, regulate membrane fluidity and permeability and probably play a role in the adaptation of membranes to temperature. This structural role is often described as the ‘bulk’ function, because it is played by significant amounts of sterols and can be fulfilled by virtually any of the compounds. In plants, sterols are substrates for the synthesis of a wide range of secondary metabolites (such as cardenolides, glycoalkaloids, pregnane derivatives and saponins) to which precise physiological functions have not been assigned [3,34,36,37].

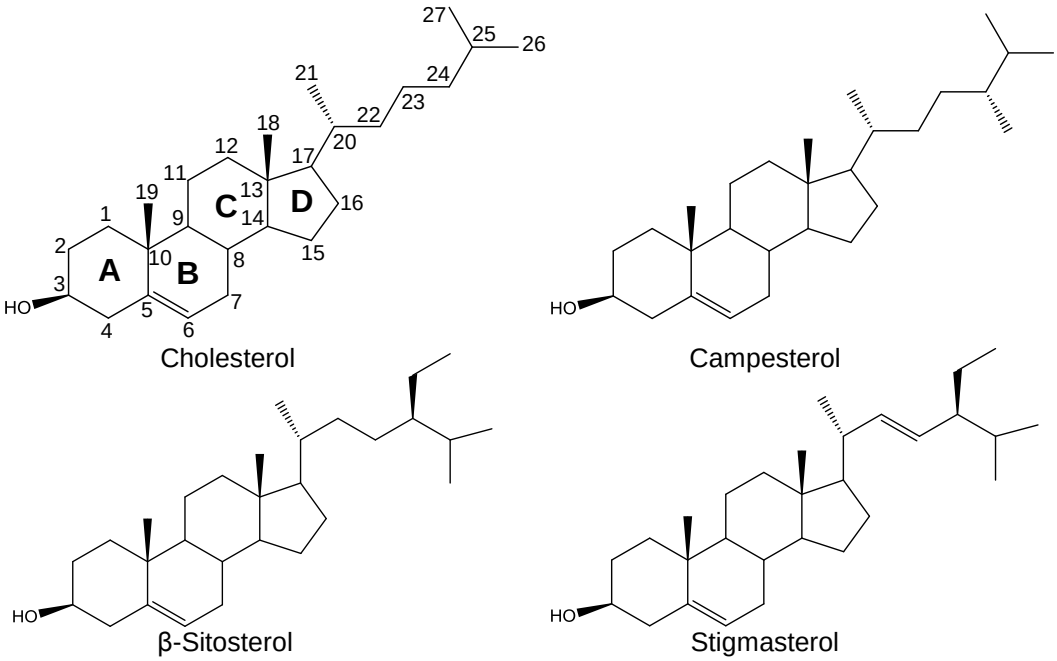


Figure 4 – Molecular structure of some sterols.

These compounds are known to be essential for all eukaryotes. In contrast to animal and fungal cells, which contain only one major sterol, plant cells synthesize a complex array of sterol mixtures in which sitosterol, stigmasterol and cholesterol often predominate. Sitosterol and cholesterol are able to regulate membrane fluidity and permeability in a similar manner to cholesterol in mammalian cell membranes. Plant sterols can also modulate the activity of membrane-bound enzymes. In contrast, stigmasterol might be specifically required for cell proliferation [3,37,38].

Active sterol synthesis occurs following seed germination to meet the needs for new membranes, the rate of sterol synthesis then gradually decreases with seed maturity. Evidence has recently emerged that sterols may play an important metabolic role in the cell proliferation process in addition to the purely structural function of controlling the physical state of membranes. For this metabolic function to take place, only a small fraction of specific sterol molecules might be necessary for cellular proliferation and differentiation. Their accumulation in seeds and oils is likely to provide a reservoir for the growth of new cells and shoots. Stigmasterol and its combination with cholesterol have in this case an important role in cell proliferation and regulation of metabolic events [36–38].

1.2.1.2 STEROLS SOURCES

Commercially, phytosterols are isolated from vegetable oils, listed in Table 1, as a co-product from the deodorization step of soybean oil, rapeseed (canola) oil, sunflower oil, corn oil, or from tall oil which is a by-product of the wood pulp industry [35,36,39].

Table 1 – Plant sterols in edible oils (g/kg) [36].

Sterols Source		Total sterols	Sitosterol	Campesterol	Stigmasterol
Corn oil	Crude	8.09-15.57	9.89	2.59	0.98
	Refined	7.15-9.52	6.9	1.58	0.76
Cottonseed	Crude	4.31-5.39	4	0.26	TR
	Refined	3.27-3.97	3.03-3.43	0.20-0.31	TR-0.04
Olive	Extra Virgin	1.44-1.50	1.18-1.21	0.05	0.01
	Pomace	2.61-2.82	2.21-2.36	0.09-0.10	0.05-0.06
Palm	Crude	0.71-1.17	0.72	0.23	0.04
	Refined	0.49-0.61	0.30-0.35	0.10-0.18	0.06-0.07
Rapeseed	Crude	5.13-9.79	2.84-3.58	1.56-2.48	0.02-0.04
	Refined	2.50-7.31	1.38-3.73	0.76-2.70	TR
Rice bran	Crude	32.25	17.45	6.58	2.52
	Refined	10.55	5.71	2.15	0.82
Soybean	Crude	2.29-4.59	1.22-2.31	0.62-0.76	0.45-0.76
	Refined	2.21-3.28	1.23-1.73	0.47-0.82	0.47-0.52
Sunflower	Crude	3.74-7.25	4.65	0.69	0.75
Tall oil	By-product	4.6-15.7	3.6-8.0	0.4-2.5	<0.1

TR- traces.

1.2.1.3 SCIENTIFIC STUDIES AND APPLICATIONS

In recent years, sterols have been investigated for its pharmacological prospects such as antiosteoarthritic, antihypercholestrolemic, cytotoxicity, antitumor, hypoglycaemic, antimutagenic, antioxidant, anti-inflammatory and Central Nervous System effects [40]. Recent studies suggested that dose intake of plant sterols of about 2g/day of stanols or sterols offer an ideal dose for lowering cholesterol levels. Also, the intake of phytosterol esters has been recommended to be taken once with each major meal, because the inhibition of biliary cholesterol absorption has been assumed to be potentiated during the food-induced emptying of the gall bladder [36]. Studies also point that dietary β-sitosterol may offer protection from chemically induced colon cancer [31,40–42].

Stigmasterol is employed in a number of chemical processes which are designed to yield numerous synthetic and semi-synthetic compounds for pharmaceutical industry [40]. It

acts as a precursor in the manufacture of synthetic progesterone, vitamin D3, as well as acting as an intermediate in the biosynthesis of androgens, estrogens, and corticoids. Research has indicated that stigmasterol may be useful in the prevention of certain cancers (ovarian, prostate, breast, and colon cancers), inhibiting cholesterol biosynthesis as well has a potential anti-osteoarthritic effect [42–44].

1.2.1.4 MARKET

According to the European Food Safety Authority (EFSA), the consumption of 1.5-3 g of plant sterols per day can significantly reduce the level of low-density lipoprotein cholesterol in individuals if consumed as part of a healthy diet. To take advantage of the cholesterol-lowering effect, an increasing number of food products with added plant sterols or plant sterol esters have become available on the EU market [45].

The European market of plant sterol compounds in 2007 was roughly estimated at about 9500 ton worth close to €160 million of which 80% were used in food products. The concentration of plant sterols in the finished products varies between 0.3% by weight in dairy products to 8% in yellow fat spreads. Given that these fat spreads were in 2005 the most common on the market commanding a 75% share of added plant sterols, it was estimated that roughly 72000 ton of yellow fat spreads with added plant sterols were sold in Europe in 2007. This indicated a market value of €700 million for yellow fat spreads in Europe [45].

According to a market report published by Transparency Market Research, the global market for phytosterols was estimated to be worth over USD 391.5 million in 2010 and expected to cross USD 887.8 million by 2018. Europe is the biggest market for phytosterols, accounting for approximately 51% of the global market in 2011, followed by North America. The favorable regulatory condition in the western world is propelling the growth of this market in the region.

1.3 EXTRACTION TECHNIQUES

The extraction and recovery of a solute from a solid matrix can be regarded as a three-stage process: local desorption of the solutes and their solubilization; internal solute diffusion through the particle until its surface; external solute diffusion through the film. In order to obtain quantitative and reproducible recoveries, careful control and optimization of each step is required; in particular, the collection of the extract needs to be carefully controlled as it is often neglected when compared to the extraction step. In

practical environmental applications (*e.g.* extraction of pollutants from soils and sediments), the first step is usually rate-limiting, as solute–matrix interactions are difficult to overcome. However, for other matrices (*e.g.* plant materials), the extraction rate may be limited by either the solubilization or the diffusional step. As a consequence, the optimization strategy will strongly depend on the nature of the matrix to be extracted [46]. The quality of an extract depends on which part of the plant is used as starting material (origin, degree of processing, moisture content and particle size), the type of solvent and the extraction procedure itself. The extraction type, time, temperature, nature and concentration of solvent (Table 2) as well as the solvent-to-sample ratio will affect the quantity and the composition of secondary metabolite composition, where the solvent to dry matter ratio of 10:1 (v/w) has been considered ideal by earlier reported studies [47].

Table 2 – Some solvents used for active component extraction [47].

Water	**Ethanol**	**Methanol**	**Chloroform**	**Ether**	**Acetone**
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonols	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

A good solvent includes low toxicity, low boiling point, rapid mass transfer of the solutes, preservative action and inability to cause the extract to complex or dissociate. In order to choose a solvent wisely one must take into account the quantity of phytochemical to be extracted; the rate of the extraction; the diversity of different compounds as well as its inhibitors; the ease of handling; its toxicity in the bioassay process; and the extractants health hazardous potential [47].

1.3.1 SOXHLET EXTRACTION

Conventional Soxhlet extraction has been the most used extraction technique worldwide for a number of decades, surpassing the performance of other alternatives and being used as an efficiency reference for comparison with new counterparts [47,48].

The Soxhlet extraction procedure and apparatus (Figure 5) is relatively simple. First the solid material containing the desired solutes is placed and packed inside a thick filter paper chamber, which is then loaded into the main chamber of the Soxhlet.

The solvent (dichloromethane, n-hexane, ethanol, methanol, or other) is put into the distillation flask and the Soxhlet extractor is then placed onto the flask and equipped with a condenser. The distillation flask is then placed on a heating mantel which will act as reboiler. The solvent is heated to reflux and steam travels to the condenser changing phase and flooding the extraction chamber, which is slowly filled with warm solvent (solubilizing the solute). When the extraction chamber is almost full, the chamber is cyclically emptied by a siphon, into the solvent flask (reboiler). During each cycle a portion of the extractives is dissolved and these cycles may be allowed to repeat many times, over hours or days.

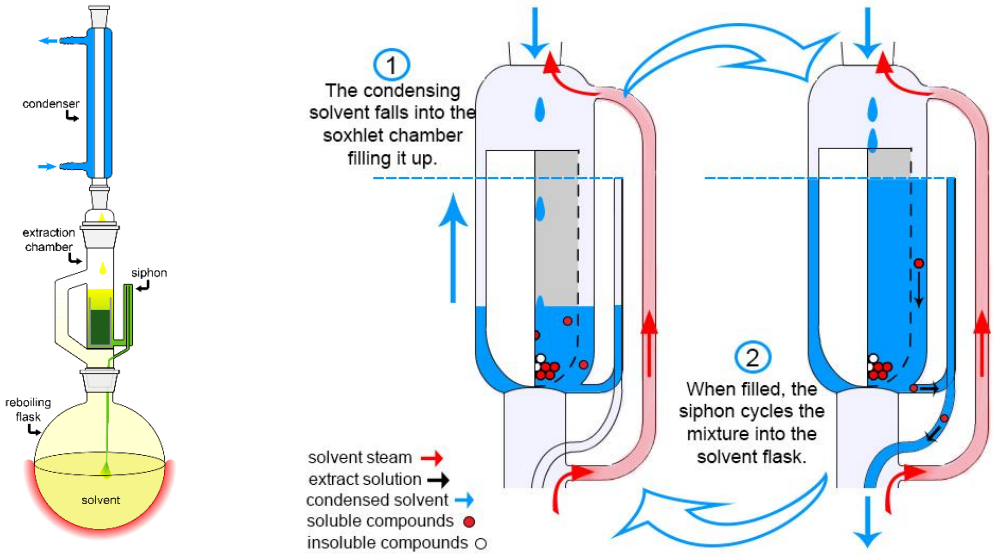


Figure 5 – (Left) Soxhlet extraction apparatus; (Right) Soxhlet cycle extraction details**.**

The choice of the extraction solvent varies with the desired extract solute characteristics, hence sequential extractions can be performed by varying the solvent according with its selectivity. The efficiency of the extraction depends on several characteristics like the packed solid matrix, particle size, solvent and temperature (this one linked to the pressure fixed in the system).

The most outstanding advantages of conventional Soxhlet are as follows: the sample is repeatedly brought into contact with fresh solvent, thereby ensuring higher mass transfer driving force; no filtration is required after the leaching step; sample throughput can be increased by simultaneous extraction in parallel, since the basic equipment is inexpensive; its simple methodology needs little specialized training.

The most significant drawbacks of Soxhlet extraction are: the extended extraction time and the large amount of used solvent (expensive disposal and environmental problems); samples are usually extracted at the solvent's boiling point for a long period of time and the possibility of thermal decomposition of the target compounds cannot be ignored; It has no agitation, which would accelerate the process; an evaporation/concentration step after the extraction is mandatory; the technique is restricted to solvent selectivity and is not easily automated [48].

1.3.2 SOLID-LIQUID EXTRACTION

Solid-liquid extraction by maceration is one of the general techniques used for the extraction of medicinal plants. The sole purpose of such basic procedure is to obtain the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent.

In the maceration process, the coarsely powdered biomass is placed in a container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent or constant agitation until the soluble matter has been extracted. The mixture is then filtered, the damp solid material is pressed, and the mixture is clarified by filtration [49].

1.3.3 SUPERCRITICAL FLUID EXTRACTION

Conventional extraction methods using liquid solvents are slow and usually require several hours or even days to effect complete extraction. They also result in dilute extracts, thus necessitating concentration of the extracted solute in the liquid solvent, and they may not result in quantitative recovery of target solutes. In certain cases, organic solvents are associated with hazardous effects. Moreover, an ideal extraction method should be rapid, yield quantitative recovery of target solute without degradation, and the solvent should be easily separated from the solute. All these facts support the development of alternative methods. Supercritical fluid extraction (SFE) technology offered scientists attractive features overcoming many of the limitations of conventional extractions [50].

A fluid is in the supercritical state when its temperature and pressure are raised above their critical values. Under these conditions, various properties of the fluid are placed between those of a gas and those of a liquid (Figure 6). Visually, the transition into supercritical state (P>Pc), will start with a well observed meniscus that with the raising temperature will be less and less distinguishable, since gas and liquid densities become

more similar. Once the critical temperature is surpassed the two phases of liquid and gas are no longer visible, hence the supercritical fluid phase is attained [51].

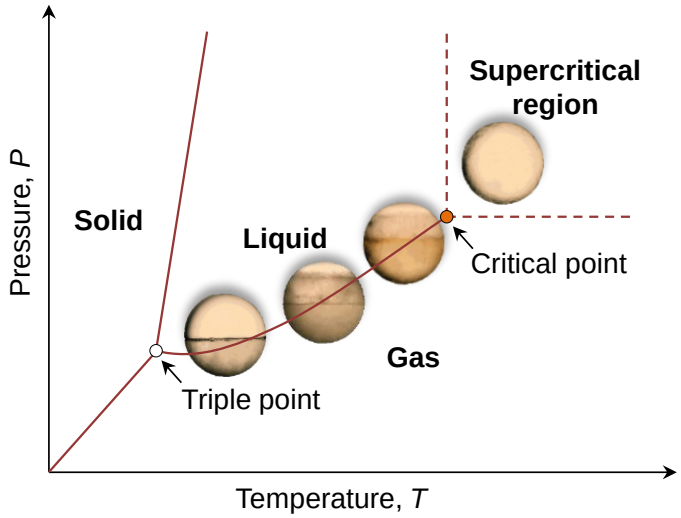


Figure 6 – Carbon dioxide pressure-temperature phase diagram with a visualization of the fluid along the vapor-liquid equilibrium line.

SFE is advantageous as a sample preparation strategy because it uses a supercritical fluid as solvent with physical properties that are a blend between gas and liquid. In reality, the strength of this resides in the fact that physical instrumental parameters can be changed to modify the chemical interactions directly, unlike any other extraction technique [52–54]. These fluids are macroscopically homogeneous but microscopically inhomogeneous consisting of solvent clusters and free volumes which result in unusual properties attributed to local density effects. As general properties, they possess high diffusive power, low viscosity, good penetration capabilities and adjustable density and selectivity to fit any particular needs [55].

Although the density of a supercritical fluid is similar to a liquid and its viscosity is similar to a gas, its diffusivity is intermediate between the two states, as can be seen in Table 3. Thus, the supercritical state of a fluid has been defined as a state in which liquid and gas are indistinguishable from each other, or as a state in which the fluid is compressible (i.e. similar behavior to a gas) even though possessing a density similar of a liquid and, therefore, similar solvating power [56,57].

Table 3 – Order of density, viscosity and diffusivity of gases, liquid and supercritical fluids [57].

Physical State	Density (ρ, g/cm^3)	Diffusivity (D_{AB}, cm^2,s)	Viscosity (μ, g.s/cm)
Gas	10^{-3}	10^{-4}	10^{-1}
Liquid	1	10^{-2}	10^{-6}
Supercritical	[0.2;0.9]	10^{-4}	10^{-3}

Because of the distinct physicochemical properties of supercritical solvents, the SFE provides several operational advantages over conventional extraction methods. Due to their low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than liquids, can diffuse easily through solid materials and can therefore give faster extraction rates. One of the main characteristics of a supercritical fluid is the possibility of modifying the density of the fluid by changing its pressure and/or its temperature. Since density is related to solubility, by altering the extraction pressure, the solvent strength of the fluid can be modified. Other advantages, compared to other extraction techniques, are the use of solvents generally recognized as safe (GRAS), the higher efficiency of the extraction process (in terms of increasing yields and lower extraction times), and the possibility of direct coupling with analytical chromatographic techniques such as gas chromatography (GC) or supercritical fluid chromatography (SFC) [57].

There is a wide range of solvent compounds that can be used as supercritical fluids (Table 4). Carbon dioxide is the most commonly used solvent for three major reasons: first, it is innocuous to human health and to environment, respecting the sustainability criteria that increasingly governs the adequacy of chemical processes; secondly its moderate critical temperature (31.3 ºC) is a key issue for the preservation of bioactive compounds in extracts; finally the extract is preserved from air, where light oxidation reactions are avoided [58].

Carbon dioxide is a gas at room temperature, so once the extraction is completed, and the system decompressed, the elimination of CO_2 is achieved, yielding a solvent-free extract. On an industrial scale, when carbon dioxide consumption is high, the operation can be controlled to recycle it. However, because of its low polarity, CO_2 is less effective in extracting more polar compounds from natural matrices. To overcome this problem, modifiers (also called cosolvents) are commonly used. Modifiers are polar compounds that, added in small amounts, can induce substantial changes of the solvent properties of neat supercritical CO_2 [57,58].

Table 4 – Critical properties of some solvents used in SFE [57].

Solvent	Critical Property			
	Temperature (ºC)	Pressure (atm)	Density ρ_{SCF} (g/mL)	Solubility σ_{SFC} ($cal^{-1/2}cm^{-3/2}$)
Carbon Dioxide	31.2	72.9	0.470	7.5
Ethane	32.4	48.2	0.200	5.8
Ethene	10.1	50.5	0.200	5.8
Methanol	-34.4	79.9	0.272	8.9
Nitrous Oxide	36.7	71.7	0.460	7.2
n-Butene	-139.9	36.0	0.221	5.2
n-Pentane	-76.5	33.3	0.237	5.1
Sulfur hexafluoride	45.8	37.7	0.730	5.5
Water	101.1	217.6	0.322	13.5

Supercritical extraction basically occurs in two steps, the solubilization of the chemical compounds present on the solid matrix and its separation into the supercritical solvent. During the extraction, the solvent flows through the packed bed, solubilizing the existing compounds present in the matrix. Afterwards the solvent exits the extractor carrying the solubilized compounds, and by pressure reduction and/or temperature increase, the extract is liberated from the solvent.

According to Brunner [59] the solubilization process of a vegetal matrix occurs in different stages. First the vegetal matrix absorbs the supercritical solvent, swelling its cellular structure, membranes and dilating the intercellular channels, leading to a drop in the resistance to mass transfer; in the meantime, dissolution of the extract occurs, and mass transfer take place from the inner matrix to its surface and solubilized compounds reach the external surface. These are finally transported from the surface to the supercritical solvent and removed from the solvent.

The SFE optimization requires the knowledge of thermodynamic data (solubility and selectivity) along with kinetic data (mass transfer coefficients). The kinetic representation of a SFE is constructed with the knowledge of its extraction curve which is normally represented in a graphic of accumulated extracted mass *versus* time of extraction. The obtained curve depends on the process parameters and the phenomena that take place in the fixed bed during the extraction process.

The extraction curve trend can easily be affected by solvent flow rate and bed particles size making it difficult to compare curves obtained from different raw materials and different equipment. However, diverse information supplied by extraction curves, like the

constant extraction rate duration, allows the comparison between different experiments with the same substrate and equipment [59].

The study of supercritical extraction curves and the knowledge of the effects of the operational variables allow the establishment of the extractor volume and solvent flow rate (Q_{CO2}). According to the literature [53,54,60,61], the overall extraction curves (OEC), are clearly divided into three periods (Figure 7) controlled by different mass transfer mechanisms:

a) Constant Extraction Rate (CER) period, where the external surface of the particles is covered with solute (easily accessible solute) and the convection is the dominant mass transfer mechanism;
b) Falling Extraction Rate (FER) period, where failures in the external surface oil layer appear and the diffusion mechanism starts, operating combined with convection;
c) Low Extraction Rate (LER) or Diffusion-Controlled (DC) period, where the external layer of oil practically disappeared and the mass transfer occurs mainly by diffusion inside the solid particles.

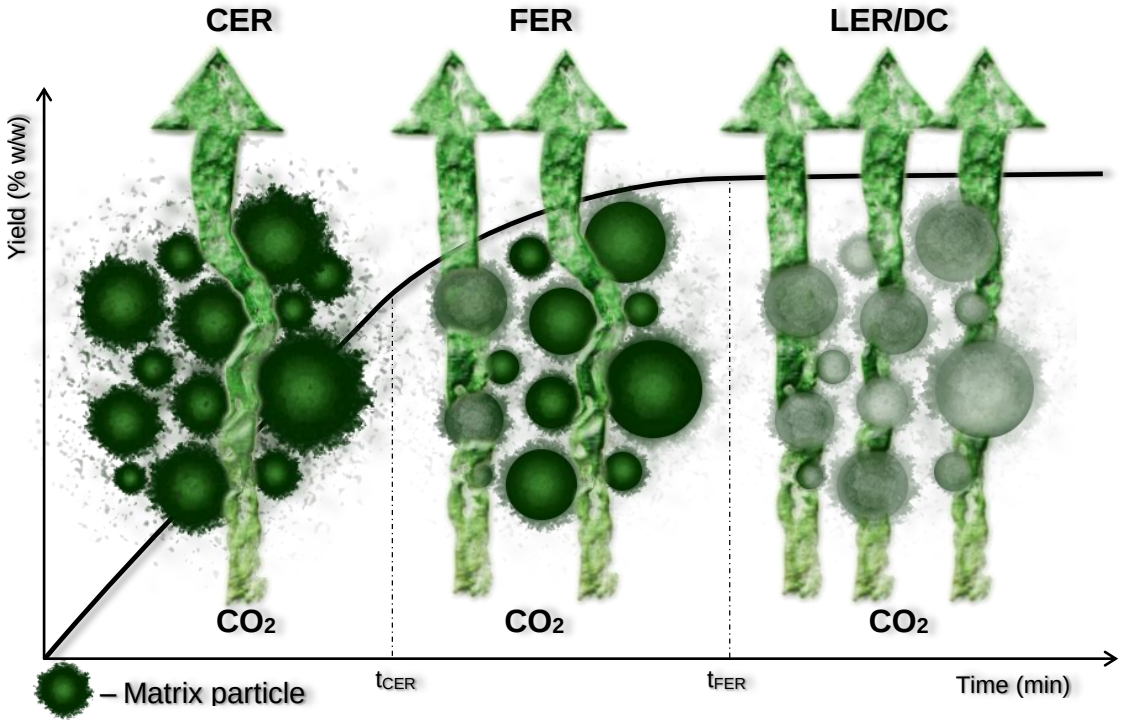


Figure 7 – Graphical illustration of the three different extraction periods and its extraction curve.

Summarily, the main advantages of SFE are:

- Ease of separation of the extracted solute from the supercritical fluid solvent by simple expansion;
- The majority of used gases are physiologically safe and inert;
- With many fluids, the separation is processed at low temperature, which is extremely important when extracting compounds from natural resources;
- Fluid proprieties vary either by the adjustment of temperature, pressure or by the addition of cosolvents, which is particularly important in the cases of solvent power and selectivity;
- The addition of cosolvents allows differential extraction within the range of nonpolar to polar solutes;
- Obtained extracts suffer minimum hydrolysis, oxidation, esterification or thermal degradation, therefore better representing the original matrix source;
- Solute diffusivity is greater when compared to liquid solvents;
- Because carbon dioxide is inert and inflammable it is wildly use as a supercritical fluid;
- Solvents can be recovered and recycled, which means low operational cost;

The main disadvantages of the SFE are as listed:

- The cost of the equipment: an industrial processing unit has an estimate cost of 1 M$ and can process 450 L for each extraction [62]. Therefore resulting extracts of low economic value and low yield are in principle not economically viable;
- Increased polar compounds are very difficult to extract even with the use of cosolvents;

1.4 MODELING

Modeling and simulation are the fundamental tools for the prediction of dynamic and equilibrium behavior, optimization of operating conditions, and scale-up of chemical plants. Modeling is fundamental in order to identify the dominant mass transfer mechanism of the SFE process. In this context SFE modeling is essential and theoretical models are highly desirable for this purpose. However, several theoretical approaches involve specific and many times complex factors that vary according to the studied case, which are frequently related with solute‑matrix interactions, flow pattern and microscopic structure of the biomass [63–68].

There are various models employed to describe the supercritical fluid extraction of oils or other compounds from plant materials. All of them consider that the particles are packed inside an extractor column, and it is usually assumed, that the solute loading in the supercritical fluid is low and, therefore, fluid density, axial dispersion, and fluid velocity remain approximately constant [69].

With the purpose of gathering information about the prevailing mass transfer mechanism of the SFE process, four models were chosen to represent and analyze the extraction curves obtained. The first two models focus on intraparticle diffusion, namely the Diffusion model for spherical particles [70] (DFM) and the Single Simple Plate model (SSPM) for slab geometry [71], while the final two models, namely the Logistic model (LM) and the Desorption model (DM), focus on the interfacial mass transfer.

The Logistic model (LM, Eq. 1) proposed by Martínez *et al.* [72] neglects axial dispersion and the accumulation in the bed, and assumes that the interfacial mass transfer of the extraction only depends on the composition of the extract along the process. A logistic equation usually applied for population growth was adopted to describe the variation of the extract composition over time:

$$\eta_{\text{total}}(t) = \frac{X_0}{e^{(btm)}}\left[\frac{1+e^{(btm)}}{1+e^{b(tm-t)}} - 1\right] \quad \text{(Eq. 1)}$$

Here, η_{total} is the total extraction yield in kg of solute per 100 kg of biomass, *t* is the extraction time in hours, and *b* and *tm* are the two parameters of the model. Parameter *tm* has a precise physical meaning, understood as the time when the extraction rate reaches its maximum value. In this model, similarly to the others presented, the mass fraction of the target species in the extractable raw material (X_0) is required to be known. X_0 (kg of solute per 100 kg of biomass) is the original extractable solute concentration in the raw material. In this work, X_0 is taken as the extraction yield determined by Soxhlet with dichloromethane.

The Desorption model (DSM, Eq. 2) proposed by Tan and Liou [73] assumes that the interfacial mass transfer is well described by a first-order kinetic expression and k_d is its desorption constant:

$$\eta_{\text{total}}(t) = \frac{A}{k_d}\left[1 - e^{(k_d B)}\right] \times \left[e^{(-k_d t)} - 1\right] \quad \text{(Eq. 2)}$$

$$A = \frac{Q_{CO_2}(1-\varepsilon)X_0\rho_s}{m_{Bio}\varepsilon\rho_{solv}}$$

$$B = \frac{\varepsilon HS\rho_{solv}}{Q_{CO_2}}$$

where Q_{CO_2} is the CO_2 mass flow rate, ε is the bed porosity, S is the extractor cross-sectional area, H is the bed height, m_{bio} is the mass of raw material in the extractor and ρ_s and ρ_{solv} are the biomass and solvent densities, respectively.

The Simple Single Plate model (SSPM, Eq. 3) presented by Gaspar *et al.* [71] assumes there is no fluid phase resistance to mass transfer, and that the process is governed by intraparticle diffusion:

$$\eta_{\text{total}}(t) = X_0\left[1 - \sum_{n=0}^{\infty} \frac{0.8}{(2n+1)^2} e^{\left(-\frac{D_m(2n+1)^2\pi^2 t}{\delta^2}\right)}\right] \quad \text{(Eq. 3)}$$

Although the effective diffusivity in the matrix, D_m ($\mathrm{m^2\ h^{-1}}$), is the adjustable constant, it was joined with the plate thickness, δ (m), so that the resulting ratio D_m/δ^2 (h^{-1}) is set as the real fitting parameter.

The Diffusion model (DFM, Eq. 4) was derived by Crank [70] for spheres, who assumed only intraparticle transport resistance and established an analogy with heat transfer by considering the solid particles as hot balls cooling in an uniform medium:

$$\eta_{\text{total}}(t) = X_0\left[1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty} \frac{1}{n^2} e^{\left(-\frac{D_m n^2\pi^2 t}{{R_p}^2}\right)}\right] \quad \text{(Eq. 4)}$$

Analogously to the SSPM, the adjustable parameter D_m ($m^2\ h^{-1}$), was joined with particle radius R_p (m) so that the resulting ratio D_m/δ^2 (h^{-1}) is set as the real fitting parameter.

The fitting adequacy of the models can be quantified by the average absolute relative deviation (AARD) defined by:

$$\text{AARD}(\%) = \frac{100}{n}\sum_{i=1}^{n}\left|\frac{\eta_{\text{total,i}}^{\text{calc}} - \eta_{\text{total,i}}^{\text{exp}}}{\eta_{\text{total,i}}^{\text{exp}}}\right| \quad \text{(Eq. 5)}$$

where n is the number of points of the cumulative curve, $\eta_{\text{total,i}}^{\text{calc}}$ and $\eta_{\text{total,i}}^{\text{exp}}$ are the calculated and experimental total extraction yields of point i, respectively.

2. EXPERIMENTAL

One of the main objectives of this work is the extraction and chemical characterization of compounds from *Eichhornia crassipes* with potential interest by SFE and Soxhlet techniques. Both results were compared in terms of yield and concentration of solutes in the extract. In order to accomplish this objective, a plan was outlined that consisted in the separation of the plant in its different morphological parts, followed by extractions and chemical characterization of the extracts, as can be seen in the flowchart plan shown in Figure 8.

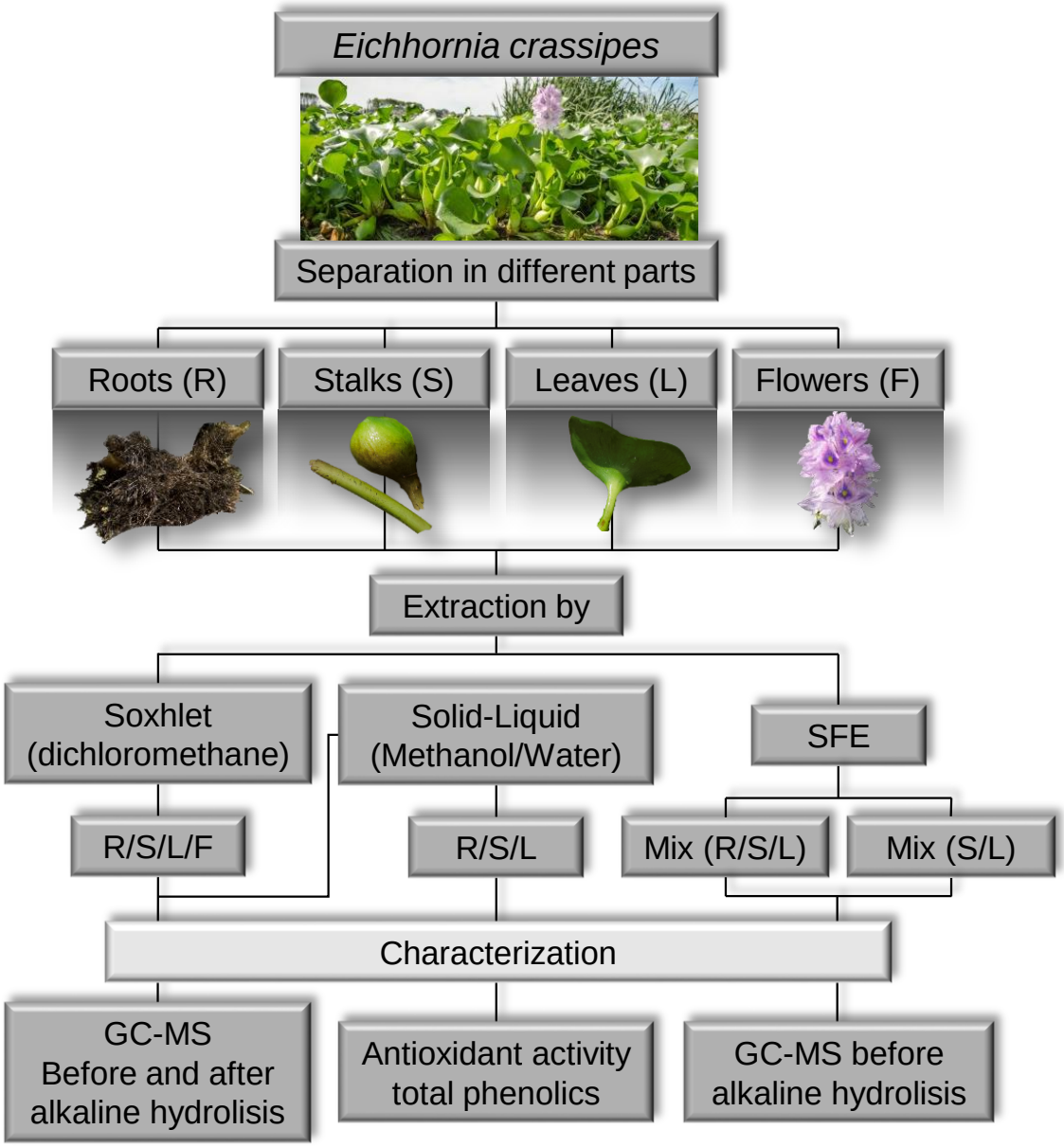


Figure 8 – Flowchart of the experimental plan.

2.1 CHEMICALS

Dichloromethane (99.99% purity), ethanol (99.99% purity) and methanol (99.99% purity) were supplied by Fisher Scientific. Gallic acid (97.5% purity), 2,2-Diphenyl-1-Picrylhydrazyl hydrate (DPPH), Folin–Ciocalteu's phenol reagent, pyridine anhydrous (99.8% purity), hexadecanoic acid (99.9% purity), nonadecan-1-ol (99% purity), 5-cholesten-3β-ol (99% purity) and vanillin (99% purity) were supplied by Sigma-Aldrich. Sodium carbonate was supplied by Panreac. Ascorbic acid (99.5% purity), trimethylsilyl chloride (99% purity), tetracosane (99% purity), hydrochloric acid (37% purity) and N,O-bis(trimethylsilyl)trifluoroacetamide (99% purity) were supplied by Fluka. Carbon dioxide was supplied by Air-Liquid (99.95% purity). Betulinic acid (98% HPLC purity), usolic acid (98% HPLC purity) and oleanolic acid (98% HPLC purity) were supplied by Aktin Chemicals. Betulonic acid (98% purity) was supplied by Chemos GmbH. Acetic acid (100% purity) was supplied by VWR International.

2.2 RAW MATERIALS

Water hyacinth (*Eichhornia crassipes*) was harvested in two different seasons from Pateira de Fermentelos's Lagoon, Aveiro, Portugal. The first harvest occurred in September 2013 and the second took place in February 2014. Part of this biomass was washed, weighted and totally dried in order to determine moisture content. The remaining biomass was washed and separated in roots(R), stalks(S), leaves(L) and flowers(F) (flowers were only captured in the first harvest). After this classification all parts of the biomass were air-dried at 55 ºC until constant weight. All dried biomass was grinded with a mill, being the final granulometry distribution (mill grade) the following: 4% (wt.) with d_p < 18 mesh; 13% (wt.) with d_p between 18 and 40 mesh; 22% (wt.) with d_p between 40 and 60 mesh; and 61% (wt.) with d_p > 60 mesh.

2.3 SOXHLET EXTRACTION

Each part of the studied biomass (roots(R), stalks(S), leaves(L) and flowers(F)) was submitted to a Soxhlet extraction with dichloromethane for a period of 8 hours in order to obtain their lipophilic fraction. Two Soxhlet extractions of *E. crassipes* mixtures were also performed for the same period of time in order to compare with the SFE results. The first mixture was composed by R/S/L (12%/24%/64%) and the second mixture was composed by S/L (35%/65%). All extractions were performed in duplicate.

The solvent was evaporated to dryness, extracts weighed, and the results expressed in weight percent (g per 100 g of dry sample).

2.4 SOLID-LIQUID EXTRACTION

The phenolic fraction of *E. crassipes* was obtained after the Soxhlet extraction. The solid biomass residues were suspended (1:100 g/mL) in methanol:water:acetic acid mixtures, 47.5;47.5;5 (v:v:v), at room temperature for 24 hours under constant stirring. This methodology was adapted from Santos et al. [74].

The suspensions were filtered and the solvent mixtures evaporated under vacuum. The evaporation process was performed at least twice in order to minimize the final acetic acid concentration and then the extracts were freeze dried and weighed.

2.5 SUPERCRITICAL FLUID EXTRACTION

Supercritical extraction experiments were performed in a spe-ed SFE apparatus (Applied Separations). In this system a cooled liquid pump is used to pressurize the liquid CO_2 in order to achieve the desired pressure of extraction, which then goes through a Coriolis mass flow meter used to measure the CO_2 flow rate. The pressurized liquid is then heated to the operating temperature in a vessel placed before the extractor column. Then, the solvent in the supercritical state flows upwards through the extractor where the biomass sample was previously loaded. Afterwards, the extract stream is depressurized in a heated backpressure regulator valve and bubbled in ethanol to capture the extract for subsequent yield quantification and GC–MS analysis. The spent CO_2 is vented to the atmosphere. The addition of cosolvent and its flow rate control is accomplished by a liquid pump (LabAlliance Model 1500) coupled to the gas line between the mass flow meter and the heating vessel placed before the extractor bed, in order to mix both CO_2 and cosolvent before feeding the extraction vessel [75]. In each run, approximately 70 g of milled *Eichhornia crassipes* was introduced in the extraction vessel. A 200 bar pressure and a constant CO_2 mass flow rate of 11 g min^{-1} were used in all extractions during six-eight hours. The list of all experiments along with their operating conditions can be found in Table 5.

After the supercritical extraction, the ethanol of the extract was firstly removed in a rotary evaporator, subjected to a stream of nitrogen in order to evaporate the excess solvent and finally put in vacuum oven at 60 ºC for 6 h so that full dryness was achieved. The extracts were weighed and the results expressed in percentage of dried biomass.

Table 5 - Operating conditions for each supercritical extraction.

SFE		Q_{CO_2} (g/min)	P (bar)	t (h)	Biomass mixture (wt.%)	T (ºC)	Ethanol content (%)
SFE-1	1a	11	200	6	R/S/L (12/24/64)	60	-
	1b			6+6		40	-
	1c			12+6			5
	1d			18+6			10
SFE-2	2a			6	R/S/L (12/24/64)	40	-
	2b			6+6			
SFE-3				8	S/L (35/65)	40	-
SFE-4						60	

2.6 EXTRACT CHARACTERIZATION

2.6.1 GC-MS ANALYSIS

GC-MS technique enables quantitative determination of the extractives and, for this reason, it was the main analytical technique employed in this study. Before GC−MS analysis, approximately 20 mg of each dried sample was converted into trimethylsilyl derivatives [76]. Each sample was dissolved in 250 µL of pyridine containing 1 mg of tetracosane (internal standard), and compounds with hydroxyl and carboxyl groups were converted into trimethylsilyl (TMS) ethers and esters, respectively, by adding 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylsilyl chloride. The mixture was maintained at 70 °C for 30 min [77].

Two aliquots of each extract were analyzed in duplicate, being the reported results the average of the concordant values obtained for each part (less than 5% variation between injections of the same aliquot and between aliquots of the same sample). GC–MS analyses were performed using a Trace Gas Chromatograph 2000 series equipped with a Thermo Scientific DSQ II mass spectrometer, equipped with a DB-1 J&W capillary column (30 m × 0.32 mm inner diameter, 0.25 µm film thickness), using helium as the carrier gas (35 cm s^{-1}). The chromatographic conditions were as follows: initial temperature: 80 ºC for 5 min; temperature rate: 4 ºC min^{-1}; final temperature: 285 ºC for 10 min; injector temperature: 250 ºC; transfer-line temperature: 290 ºC; split ratio: 1:50. Total time run of each injected sample was of 72 min. The MS was operated in the electron impact mode with energy of 70 eV and the data were collected at a rate of 1 scan s^{-1} over a range of m/z of 33–750. The ion source was maintained at 250ºC.

For quantitative analysis, the GC-MS equipment was calibrated with pure reference compounds, representative of the lipophilic extractives components (namely, vanillin, hexadecanoic acid, nonadecan-1-ol, 5-cholesten-3β-ol, betulinic acid, oleanolic acid,

betulonic acid and ursolic acid), relative to tetracosane, the internal standard used [78]. The respective multiplication factors needed to obtain correct quantification of the peak areas were calculated as an average of four GC-MS runs. The compounds were identified as TMS derivatives, by comparing their mass spectra with the equipment mass spectral library (Wiley-NIST Mass Spectral Library, 1999).
Soxhlet extractions as well as supercritical extractions were characterized by this technique and the compound contents quantification was expressed as grams per kilogram of dry weight of biomass.

2.6.2 ALKALINE HYDROLISES

Approximately 20 mg of each extract were dissolved in 10 mL of 0.5 M NaOH in 10% aqueous methanol. The mixture was heated at 100 °C, under nitrogen atmosphere, for 1 h. The reaction mixture was cooled, acidified with 1 M HCl to pH ≈ 2, extracted three times with dichloromethane, and the solvent evaporated to dryness [76,77]. After this process the resulting extracts were characterized by GC-MS. The alkaline hydrolysis reaction was performed to detect indirectly esterified compounds (e.g., triglycerides, steryl esters, etc.).
Soxhlet extracts of roots, stalks and leaves were characterized by this technique and the compound contents quantification was expressed as grams per kilogram of dry weight of biomass. Flowers were not characterized by this method due to the low quantity of extract.

2.6.3 ANTIOXIDANT ACTIVITY

Antioxidant compounds may be water-soluble, lipid-soluble, and insoluble or bound to cell walls. Free radicals play an important role in degenerative diseases and can also induce nutrition and medicine deterioration. Fortunately, formation of free radicals is controlled by a variety of systems which are called "antioxidants". When antioxidants diminish or oxidants attack cells, free radicals induce oxidative damages [79].
The antioxidant activity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging methodology [80]. It can be measured in terms of hydrogen donating or radical scavenging ability, using DPPH$^{\bullet}$ as a stable radical [81]. To evaluate the antioxidant activity using DPPH$^{\bullet}$; the compounds or extracts react with it and the reduction of DPPH$^{\bullet}$ is followed by monitoring the decrease of its

absorbance at a characteristic wavelength during the reaction. Because of a strong absorption band at 515-528 nm, when DPPH• accepts an electron or a free radical species it loses this band which results in the discoloration from a deep violet color to a colorless or pale yellow (Figure 9). This feature allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at a given wavelength [82]. In this assay, antiradical compounds induce DPPH• solution discoloration, which were measured at 517 nm.

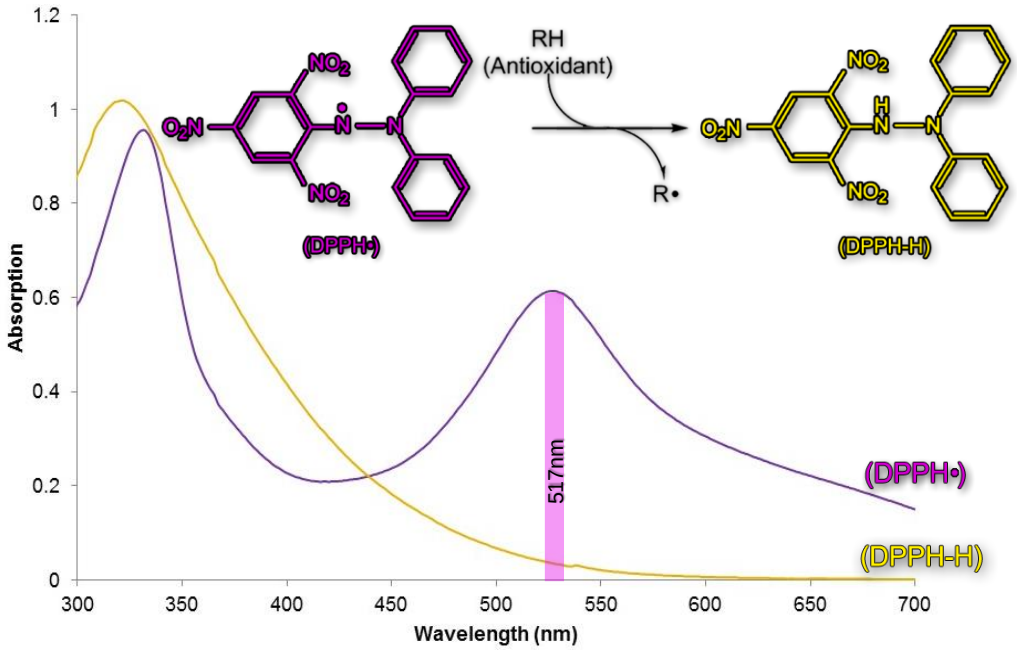


Figure 9 – Reaction between free radical DPPH• and antioxidant (RH) to form DPPH-H, along with the color shift at 517nm wavelength.

In test tubes, 0.25 mL of DPPH• with a concentration of 0.8 mM in methanol was added to accurately weighed aliquots of the extracts (roots, stalks and leaves) dissolved in 3.75 mL of the used solvent, corresponding to a concentration range of 0.025-0.25, 0.05-0.5, and 0.25-2.5 mg/mL, for leaves, stalks and roots extracts respectively. After mixing, the samples were maintained in the dark, at room temperature, for 30 minutes. The absorbance was measured at 517 nm using an UVmini-1240 spectrophotometer (Shimadzu) and compared with a control without extract. A blank was prepared for each sample using the solvent instead of the DPPH• solution. Ascorbic acid was used as reference compound.

The antioxidant activity was expressed as a percent inhibition of DPPH• radical, and was calculated by:

$$Scavenging\ activity\ (\%) = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100\% \quad \text{(Eq. 6)}$$

The obtained results were expressed as IC_{50} values (inhibition concentration value) which translate in the required concentration for a 50% reduction or inhibition of the DPPH• activity (color). These values were determined from the plotted graphs of scavenging activity against the concentration of the extracts, and are expressed in μg/mL. The antioxidant activity was also expressed in g of ascorbic acid equivalents (AAE) per g of biomass or per g of extract [83,84].

The IC50 was calculated graphically using a calibration curve in the linear range by plotting the extract concentration *vs* the corresponding scavenging effect. The antioxidant activity was also expressed as the antioxidant activity index (AAI), which was calculated as follows [85]:

$$\mathrm{AAI} = \frac{final\ concentration\ of\ DPPH^{\bullet}\ (\mu g/mL)}{IC_{50}\ (\mu g/mL)} \times 100\% \quad \text{(Eq. 7)}$$

The AAI was calculated considering the mass of DPPH• and the mass of the tested compound in the reaction, resulting in a constant for each compound, independent of the concentration of DPPH• and sample used. It is considered that plant extracts show “poor” antioxidant activity when the AAI value is lower than 0.5, “moderate” antioxidant activity when the AAI value is between 0.5 and 1.0, “strong” antioxidant activity when the AAI value is between 1.0 and 2.0, and “very strong” when the AAI value is higher than 2.0 [85]. The assays were carried out in quadruplicate and all the samples and standard solutions, as well as the DPPH• solutions, were prepared daily.

2.6.4 TOTAL PHENOLIC CONTENT (TPC)

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method [80]. Briefly, 2.5 mL of Folin-Ciocalteu reagent, previously diluted with water (1:10, v:v), and 2 mL of aqueous sodium carbonate (75 g L^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of water/methanol solution (1:1), corresponding to concentration ranges between 35 and 500 μg of extract mL^{-1}. Each mixture was kept for 5 min at 50 ºC and, after cooling, the absorbance was measured at 760 nm, using a UV-vis V-530 spectrophotometer (Jasco, Tokyo, Japan).

The TPC was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (1.5 - 60 µg/mL) and expressed as mg $_{\text{gallic acid equivalent (GAE)}}$/g $_{\text{dry extract}}$. The analyses were carried using three aliquots of each extract, measured in triplicate, and the average value was calculated in each case [74].

3. RESULTS AND DISCUSSION

3.1 EVALUATION OF *EICHHORNIA CRASSIPES*'S POTENTIAL

The valorization of *Eichhornia crassipes* or any plant biomass requires the detailed knowledge of its chemical composition. Considering the lack of detailed information about the extractives composition reported in the literature for *Eichhornia crassipes*, this thesis started with the chemical characterization of the lipophilic and polar (hydrophilic) fractions for each morphological part of the plant. Such fractions were obtained by Soxhlet with dichloromethane and solid-liquid extraction with methanol:water:acetic acid, respectively. The obtained extracts were characterized by GC-MS, techniques and the antioxidant capacity and total phenolic content were also determined.

3.1.1 EXTRACTION YIELDS

Regarding the extractives obtained from the different morphological parts of *E. crassipes*, Table 6 presents the yields values of the Soxhlet, SLE and their sum. Upon inspection of the total yields, it is noticeable that different morphological parts of the plant lead to very distinct amount of extractives. Stalks were the richest source of extractives, with 31.49% (wt.), followed by leaves with 24.23% (wt.) and roots 11.12% (wt.). Despite providing the highest lipophilic yield, flowers were not able to be compared with the other plant sections in terms of total yield since the contribution of the SLE could not be scored due to an insufficient amount of mass after Soxhlet extraction.

Taking into account that Soxhlet extraction with dichloromethane aimed at the removal of lipophilic solutes and that SLE was performed afterwards and targeted the hydrophilic extractives of the plant, informative insights on the chemical nature of *E. crassipes* extractives can be unveiled from the two middle columns of Table 6. While the hydrophilic extractives represent around 6 to 10 times the amount of lipophilic solutes in each part of the plant, their relative proportions follow a similar trend as the plant is analyzed upward. Accordingly, if Soxhlet extraction results are considered, an increment of nearly 1% in the yield is observed as roots, stalks, leaves and flowers are extracted, by this order. The same trend is observed in polar extractives, though its evolution is not linear. In this respect, stalks are the major source of polar extractives among the plant sections considered, with $\eta_{\mathrm{SLE}} = 28.84$ (wt.%). In the whole, the results from Table 6 evidence that very distinct extraction yields should be expected from distinct plant

sections, and that the polarity of the solvent can have a significant impact on the attained yields.

The yield difference between the upper part of the plant and roots may be related with their function. Roots are the main organ for the uptake of water and inorganic nutrients, and often serves as an energy storage in the form of polysaccharides such as starch and inulin [3], which can translate in a poor fraction of lipophilic compounds within the roots.

Table 6 - Extraction yields of different morphological parts of *E. crassipes* after consecutive extractions with dichloromethane (Soxhlet) and methanol:water:acetic acid mixture (SLE). Results are expressed in terms of the mean plus the respective standard deviation.

Plant section	$\eta_{\mathbf{Soxhlet}}$ (wt.%)	$\eta_{\mathbf{SLE}}$(wt.%)	$\eta_{\mathbf{Total}}$ (wt.%)
Roots	1.12 ± 0.05	10.00 ± 1.41	11.12±1.41
Stalks	2.65 ± 0.09	28.84 ± 1.46	31.49±1.46
Leaves	3.30 ± 0.14	20.93 ± 1.18	24.23±1.19
Flowers	4.45 ± 0.10	n.a.	4.45 ± 0.10

3.1.2 LIPOPHILIC EXTRACTS

The lipophilic extracts obtained by Soxhlet were analyzed by GC-MS, being the results of this measurement provided in Table 7. Twenty nine compounds have been identified (Figure 10), representing 22% to 38% (wt.) of the composition in these extracts. The most representative compounds present in the extracts were **1** glycerol, **10** hexadecanoic acid, **16** linoleic acid, **17** linolenic acid, **26** cholesterol, **27** methylcholesterol, **28** stigmasterol and **29** β-sitosterol. In fact, the identified compounds may be grouped into three dominant molecule families: sterols (ST), fatty acids (FA), long chain aliphatic alcohols (LCAA) and aromatic compounds (AC).

A noteworthy aspect devised from the comparison of GC-MS results regarding the different vegetable parts is that almost all identified compounds appeared in all morphological parts of the plant, though remarkable, unequal abundances are observed for some of these parts. For instance, two clear-cut examples of variations in extracts concentration are provided by glycerol and linolenic acid compounds, which values vary between 41-864 and 3-1149 mg/kg$_{\mathbf{sample}}$ respectively, if roots or leaves extracts are considered.

Table 7 – Lipophilic components (mg/kg of dry biomass) identified in the dichloromethane extract of *E. crassipes*, before alkaline hydrolysis.

Peak	Compounds	R_T (min)	Family	Roots	%	Stalks	%	Leaves	%	Flowers	%
1	Glycerol	13.16	FA	41±5	0.37	882±49	3.33	864±42	2.62	536±33	1.21
2	Nonanoic acid	15.06	FA	16±1	0.14	TR	-	TR	-	TR	-
3	Vanillin	19.40	AC	16±1	0.14	6±1	0.02	7±1	0.02	9±3	0.02
4	Vanillic acid	26.52	AC	11±1	0.10	55±1	0.21	41±5	0.13	167±5	0.38
5	Nonanedioic acid	27.59	FA	20±1	0.18	183±10	0.69	88±8	0.27	210±4	0.47
6	Tetradecanoic acid	28.94	FA	47±2	0.42	43±1	0.16	85±7	0.26	40±1	0.09
7	Pentadecanoic acid	31.34	FA	55±5	0.49	48±2	0.18	27±2	0.08	119±1	0.27
8	Hexadecanol	31.86	LCAA	81±6	0.72	65±3	0.25	79±6	0.24	33±0	0.07
9	Palmitoleic acid	33.29	FA	12±3	0.11	15±1	0.06	34±3	0.10	TR	-
10	Hexadecanoic acid	33.65	FA	605±31	5.40	1107±11	4.18	1242±82	3.76	2899±99	6.51
11	Ferulic acid	34.26	FA	4±1	0.04	46±1	0.17	66±12	0.20	42±3	0.09
12	Octadecenol	35.57	LCAA	TR	-	TR	-	71±5	0.21	TR	-
13	Heptadecanoic acid	37.59	LA	42±3	0.38	28±2	0.11	31±1	0.10	60±2	0.13
14	Octadecanol	36.27	LCAA	41±3	0.37	32±4	0.12	30±2	0.09	21±1	0.05
15	Phytol	36.68	LCAA	2±1	0.02	8±2	0.03	135±25	0.41	6±1	0.01
16	Linoleic acid	37.04	FA	11±2	0.10	46±8	0.17	566±43	1.72	55±3	0.12
17	Linolenic acid	37.11	FA	3±0	0.03	20±6	0.08	1149±132	3.48	38±1	0.08
18	Cis-oleic acid	37.22	FA	22±1	0.20	18±2	0.07	91±11	0.27	38±1	0.09
19	Rrans-oleic acid	37.38	FA	14±1	0.13	10±3	0.04	80±10	0.24	15±1	0.03
20	Octadecanoic acid	37.90	FA	116±2	1.04	111±9	0.42	87±9	0.26	324±5	0.73
21	Eicosanol	40.36	LCAA	17±1	0.15	7±1	0.03	10±1	0.03	17±1	0.04
22	Eicosanoic acid	41.56	FA	26±1	0.23	36±2	0.14	297±13	0.90	TR	-
23	1-monohexadecanoin	45.00	FA	37±3	0.33	237±10	0.89	372±5	1.13	382±17	0.86
24	Docosanoic acid	45.59	FA	19±1	0.17	19±8	0.07	15±3	0.05	256±9	0.58
25	1-mono octadecanoin	47.78	FA	TR	-	TR	-	103±10	0.31	351±17	0.79
26	Cholesterol	53.98	ST	360±9	3.21	694±32	2.62	891±64	2.70	462±36	1.04
27	Methylcholesterol	56.03	ST	343±19	3.06	496±31	1.87	974±182	2.95	637±48	1.43
28	Stigmasterol	56.71	ST	728±38	6.50	4014±188	15.15	4437±274	13.45	1271±81	2.86
29	β-sitosterol	57.89	ST	677±38	6.04	772±46	2.91	1051±62	3.18	1990±110	4.47
Families of compounds											
Sterols (ST)				2108	18.82	5976	22.55	7353	22.28	4360	9.80
Fatty acids (FA)				1173	9.74	2849	10.75	5198	15.75	5366	12.06
Long chain aliphatic alcohols (LCAA)				141	0.54	113	0.43	326	0.99	77	0.17
Aromatic compounds (AC)				27	0.24	61	0.23	49	0.15	176	0.40
Not identified + Not Detected				7751	69.21	17501	66.04	20074	60.83	34521	77.58
Total				11200		26500		33000		44500	

All values are expressed as mean ± standard deviation (n = 3)
Abbreviations: TR – traces; R_T – Retention time.

Despite the referred variation in the concentrations, sterols are clearly the most abundant family of compounds in all extracts (except flowers), with concentrations varying from 18.82 to 22.55% (mg/(100mg$_{extract}$)). Moreover, the major relevance of this family within the composition of the lipophilic extracts is linked to the abundance of two compounds, β-sitosterol and stigmasterol, which represent 67% to 80% of the total identified sterols.

Additionally, special attention should be drawn to the values attained by stigmasterol concentration, namely stalks and leaves extracts with 13.45% and 15.15% (wt.) respectively. In order to give proper credit to these values, it should be stressed that the richest sources of stigmasterol, among the species typically known for their edible oils found in Table 1, are crude rice bran and corn oil with concentrations of 0.25% and 0.10% (wt.) respectively [36].

In this work an alkaline hydrolysis has been also performed in order to detect possible esterified compounds in the extract. An increase in the amount of extractives was detected, particularly fatty acids (hexadecanoic, linolenic, octadecanoic, eicosanoic and ferulic acids) and long chain aliphatic alcohols (octadecenol and phytol), thus confirming the presence of esterified components. However, sterols quantities remained approximately unchanged.

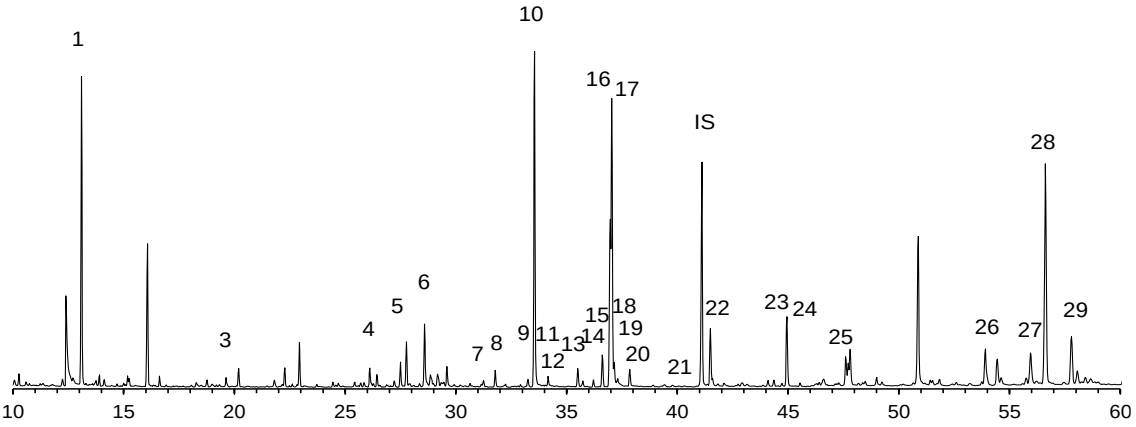


Figure 10 – Ion chromatogram of the derivatized Soxhlet extract of *E. crassipes* of leaves; IS- Internal standard.

3.1.3 HYDROPHILIC EXTRACTS

Although full characterization of the hydrophilic extracts of *E. crassipes* was impossible to be performed under the scope of this thesis, these were tested in terms of antioxidant activity and total phenolic content (TPC). To the best of our knowledge, such measurements were not yet reported for the different parts of this species.

The **antioxidant activity** was evaluated by DPPH$^{\bullet}$ radical scavenging method. Table 8 presents the results for the studied extracts, expressed in terms of the amount of extract required to reduce the DDPH$^{\bullet}$ concentration by 50% (IC_{50}), as well as in terms of ascorbic acid equivalents (AAE) on a dry biomass basis (mg AAE/g_{sample}).
Comparing the obtained antioxidant activity results, significant remarks can be observed depending on the selected plant part. Leaves present the highest antioxidant activity, followed by stalks and roots. On an ascorbic acid equivalent basis, hydrophilic extracts of leaves exhibit the highest antioxidant activity performance, with 4.86 mg AAE/g_{sample}, which is approximately 2.4 times greater than the value observed on the stalks extracts (2.04 mg AAE/g_{sample}). On the other hand, roots antioxidant activity results revealed a very poor performance. This difference can be noticed either by its extreme low value of 0.11 mg AAE/$g_{sample,}$ or by the greater order of magnitude of its IC_{50} value in comparison with leaves and stalks counterparts: 2340.91 µg/mL against 370.36 µg/mL and 113.44 µg/mL, respectively.

If the antioxidant activity results presented in Table 8 are converted into an antioxidant activity index (AAI) proposed by Scherer and Godoy [85], a direct and independent qualitative appreciation of all the studied extracts may be accomplished. Figure 11 presents the antioxidant activity results in terms of AAI scores, where different qualitative antioxidant regions are also identified. Upon examination of the graphic it is possible to confirm that the weakest antioxidant activity, obtained by roots, represents an AAI score of 0.17 that falls in the category of “poor”. Regarding the AAI scored by stalks of 1.08, this places stalks extract in the frontier between “moderate” and “strong”. Remarkably, the AAI value attained by leaves extracts indicated a value of 3.53, which falls in the region of “very strong”. As a result, and despite SLE stalks yield being higher than leaves counterpart (29 wt.% *vs.* 20 wt.%, see Table 6), leaves extracts are a much more promising source of antioxidants.

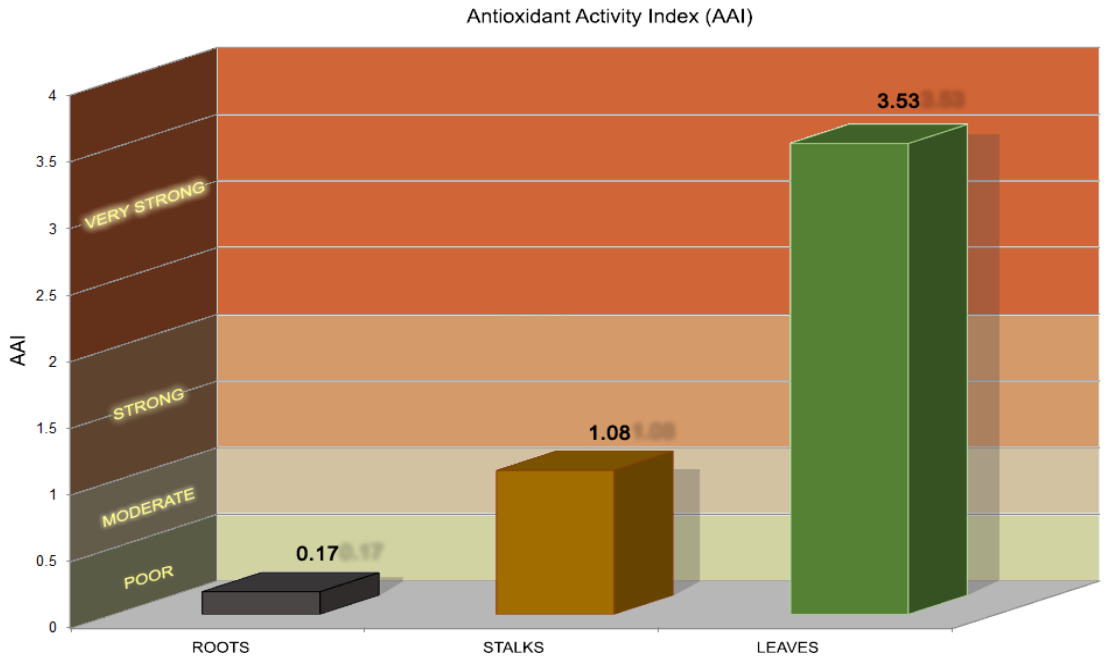


Figure 11 – Antioxidant Activity Index for roots, stalks and leaves extracts.

Table 8 – Antioxidant activity of the extracts of different morphological parts of *E. crassipes* measured by DPPH· radical scavenging, expressed as IC_{50} values, and ascorbic acid equivalents.

Biomass	IC_{50} (µg/mL)	mg AAE/g_{sample}
Ascorbic acid	2,55 ± 0,05	-
Roots	2340,91 ± 67,14	0,11 ± 0,00
Stalks	370,36 ± 7,70	2,04 ± 0,04
Leaves	113,44 ± 3,95	4,86 ± 0,17

The **total phenolic content (TPC)** was determined by Folin-Ciocalteu method according to the linear equation y=11.717x + 0.0183 (R^2=0.9958) obtained by the calibration curve as galic acid equivalents (mg GAE/$g_{extract/sample}$). TPC values of 21.88, 14.49 and 38.28 mg GAE g^{-1} in roots, stalks and leaves, respectively, clearly demonstrate that leaves are the morphological part with higher phenolic content (Table 9). When expressed on mg GAE/g of used sample, the difference between the extracts becomes more explicit, as a result of extraction yields values, where leaves TPC content doubles its value when compared to stalks, and quadruplicates when compared with TPC roots value. It is worth mentioning that this is the first study reporting phenolic content of all different morphological parts of *E. crassipes*, and that leaves TPC value is in accordance with values reported in the literature [25].

Table 9 – Total phenolic content of the extracts of different morphological parts of *E. crassipes*.

Sample	Total phenolic content	
	(mg GAE/$g_{extract}$)	(mg GAE/g_{sample})
Roots	21.88 ± 0.27	2.21 ± 0.03
Stalks	14.49 ± 0.18	4.29 ± 0.05
Leaves	38.28 ± 0.42	8.49 ± 0.09

Eichhornia crassipes characterization revealed a strong yield composition in hidrophilic content when compared with its lipophilic counterpart. Leaves presented a “very strong” antioxidant activity index value, and total phenolics content were in accordance with the reported values found in the literature [25]. It is worth noting that lipophilic chemical characterization revealed a high quantity of stigmasterol in leaves and stalks, making this biomass a good source for this type of sterol, presenting values above all the sources listed in Table 1. Stigmasterol is, as stated previously, used in the production of synthetic progesterone, androgens, estrogens, corticoids and vitamin D3. Researches also show that it can be useful in cancer prevention and in the inhibition of cholesterol biosynthesis. This valuable and potential high source of stigmasterol propelled further investigation on its lipophilic extraction, in this case, a more environment friendly, efficient, (selective and yield wise), method of extraction, the supercritical fluid extraction.

3.2 SUPERCRITICAL FLUID EXTRACTION (SFE)

Conventional extraction methods using liquid organic solvents can be slow, resulting in dilute extracts that require additional work to achieve desired concentration. In certain cases, solvents are associated with hazardous effects. Moreover, an ideal extraction method should be rapid, yield quantitative recovery of target solutes without degradation, and the solvent should be easily separated from the solute. Supercritical fluid extraction (SFE) overcomes many limitations of conventional extraction methods.

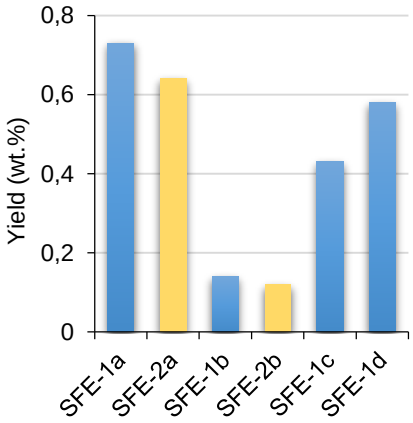
Supercritical fluid extraction in this particular case used carbon dioxide as solvent and ethanol as cosolvent in the preliminary study. Due to the low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than liquids, can diffuse easily through solid materials and can therefore give faster extraction yields. One of the main characteristics of a supercritical fluid is the possibility of modifying the density by changing its pressure and/or its temperature, with significant impact on solubility and even selectivity.

3.2.1 PRELIMINARY STUDIES

Preliminary sequential supercritical extractions (SFE1 and SFE2) of mixtures of roots, stalks and leaves of *E. crassipes* were carried out according to the experimental conditions described previously in Table 5. Soxhlet extraction of the same biomass mixture was also carried out. These extractions provided data regarding the comparison between the two extraction techniques and also valuable feedback on the performance of the extractions.

Table 10 - Extraction yields for the preliminary SFE and stigmasterol concentration on the extract. See experimental conditions in Table 5.

Extraction Type	η (wt.%)	$\eta_{Stigmasterol}$ (wt.%)	% Stigmasterol mg/(100mg$_{extract}$)
Soxhlet	2.88	0.36	13.02
SFE-1a	0.73	0.22	30.64
SFE-1b	0.14	-	-
SFE-1c	0.43	-	-
SFE-1d	0.58	0.02	2.84
SFE-2a	0.64	0.20	31.45
SFE-2b	0.14	0.04	32.02



Although Soxhlet extraction presented a superior total yield value than its SFE counterpart, its stigmasterol yield was relatively close for both types of extractions (Table 10). Furthermore, by comparing the stigmasterol concentration value attained by the SFE-1 or SFE-2 runs (*ca.* 30% mg/(100mg$_{extract}$)) with that obtained by Soxhlet extraction (*ca.* 13% mg/(100mg$_{extract}$)), one can conclude that the SFE was widely more selective to this particular molecule.

Regarding the observed yield decrease from SFE-1a to SFE-1b and also from SFE-2a to SFE-2b in Table 10, these values indicate a possible tendency toward the depletion of the easily accessed lipophilic extracts. This decrease can be translated as an inversion of the dominant mass transfer mechanism. In other words, while convection commands the mass transfer rate within the early stages of the extraction, (due to the easily accessible solute at surface), with its depletion, solute diffusion through the solid particles now becomes the new dominant mass transfer driving force in the SFE. Comparing the obtained yields of extractions SFE-1a and SFE-2a (0.73% and 0.64% (wt.) respectively), we can state that a temperature decrease of 20ºC between them resulted in a minor yield reduction. This small difference rises awareness that possibly

the extraction temperature may have little influence on the overall and stigmasterol extraction yields.

Moreover, the use of cosolvent in the extractions SFE-1c (40ºC; 5% EtOH) and SFE-1d (40ºC; 10% EtOH) resulted in a total yield value increase of 0.15% (wt.) comparatively. These overall yields exposed, as anticipated, that the obtained extracts had a high percentage of hydrophilic compounds and that the biomass at this stage had been exhausted from its lipophilic fraction.

Additionally, GC-MS analysis of the stigmasterol of SFE-1a and SFE-1d (both extractions were chosen based on their high yield values) indicated that it was the most abundant compound in the SFE-1a extract with a remarkable concentration of 30.6% mg/(100$mg_{extract}$). On the other hand, quantitative analysis of SFE-1d extract revealed a lower content in lipophilic compounds, and a higher content in hydrophilic compounds. Stigmasterol concentration of SFE-1d presented a value of 2.84% mg/(100$mg_{extract}$) which was a clear indicator of lipophilic depletion and low selectivity due to the use of ethanol as cosolvent. Stigmasterol selectivity falls (as well as its concentration in the extract) due to the increased solubilization of other competing (higher polar) molecules, rendering the use of ethanol as cosolvent not suitable for the stigmasterol extraction.

In sum, the results of this preliminary study exposed key aspects of the supercritical extraction of this biomass matrix, specifically, the applicability of cosolvent and the chemical content of the extracts. In that sense, we now know that the use of ethanol as a modifier would increase its hydrophilic fraction, lowering its selectivity, impoverishing the final extract in stigmasterol, lowering its partial yield and, therefore, its concentration.

Finally, regarding the SFE-2 extraction, and bearing in mind that both sequential steps SFE-2a and SFE-2b were performed under the same conditions, it is known from Table 10 and from Figure 12 that the second step (SFE-2b) resulted in an extreme low contribution to stigmasterol concentration as well as the total and stigmasterol yield values (see the plateau between 6 and 12 hours of extraction).

At this stage it is necessary and convenient to study the SFE along time in more detail in order to observe its evolution and disclose the main mechanisms involved.

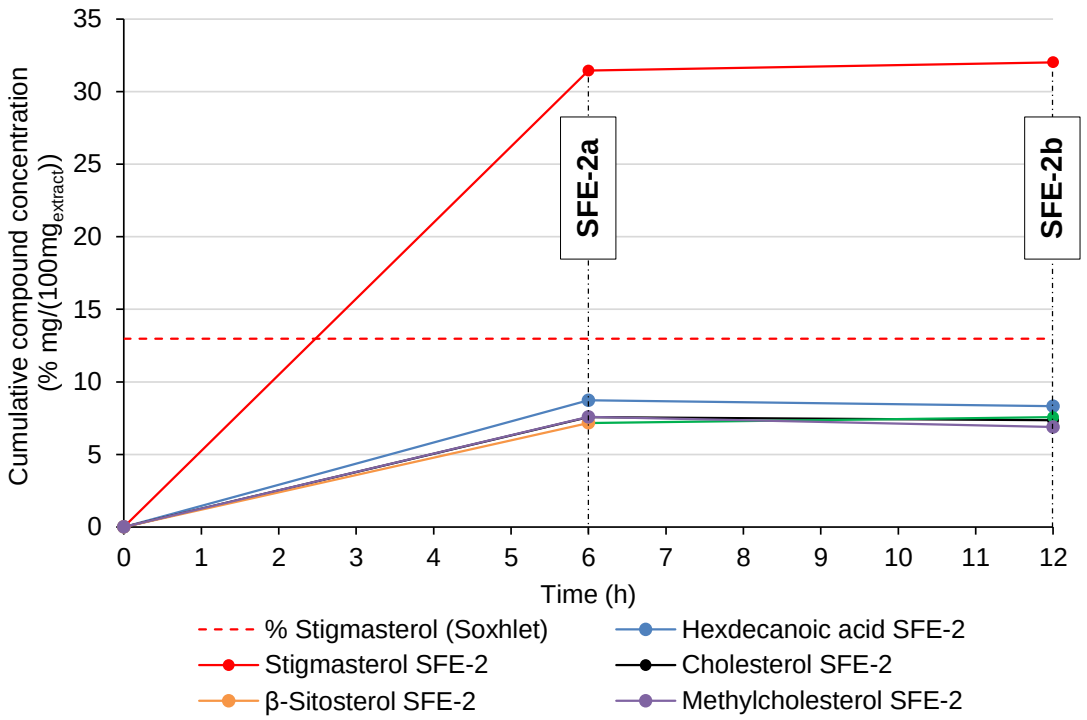


Figure 12 – Cumulative concentration of several compounds in the supercritical extracts of SFE-2 run. The Soxhlet yield in stigmasterol is also shown.

3.2.2 OVERALL EXTRACTION CURVES (OEC)

As stated previously, stalks and leaves presented higher extraction yields and higher stigmasterol content than roots. Furthermore, and because SFE was highly selective for this specific molecule, the consumed biomass used in the measurement of the overall extraction curves (OEC) is now composed by a mixture of 35% of stalks and 65% of leaves, which means that from an entire plant only roots were dismissed. This biomass was harvested in February of 2014 and its Soxhlet extraction was also performed and analyzed in order to have a direct comparison between both extraction techniques and harvest seasons for the preliminary and OEC study.

Two supercritical extractions, SFE-3 and SFE-4, were carried out with the same amount of biomass (70 g) at different temperatures, 40 and 60ºC, respectively, while the remaining conditions were maintained constant (see Table 5). The extracts of both SFE-3 and SFE-4 were collected during 8h and their composition analyzed by GC-MS. Figure 13 shows the supercritical extraction curves for SFE-3 and SFE-4 runs plotted in terms of total and stigmasterol yield.

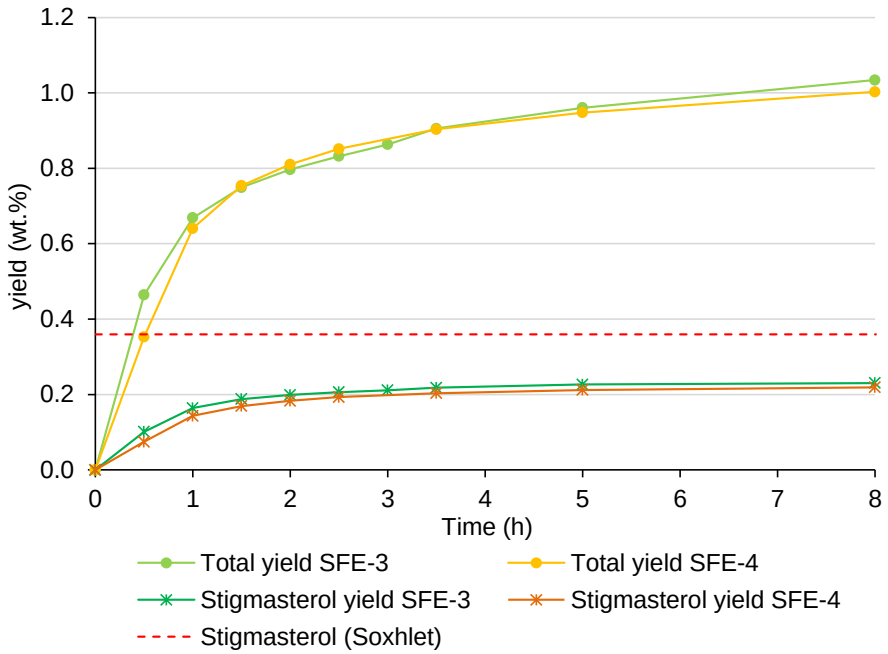


Figure 13 – Supercritical extraction curves of SFE-3 and SFE-4 runs in terms of total and stigmasterol yields.

Considering Figure 13, temperature has little impact on the total extraction yield (1.03 and 1.00 % (wt.)) and stigmasterol yield (0.23 and 0.22% (wt.)). Furthermore, Figure 13 also clarifies the trends of the different extraction periods, where two different mass transfer mechanisms clash. In the early stage [0-1h], convection is dominant and this ruling translates as a constant extraction rate period. After this period, both yields start to diminish and convection is no longer dominant and intraparticle diffusion starts to take part in the process. This period begins after the first hour of extraction and ends at the second hour. From this moment on, mass transfer is ruled by the diffusion occurring inside the solid biomass matrix.

Regarding the stigmasterol extraction curves, these perform analogously to the previously described total yield. Despite Soxhlet extraction yield (3.01 wt.%) being three times higher than its SFE counterpart (*ca.* 1 wt.% for SFE-3 and SFE-4), and stigmasterol Soxhlet yield (0.36 wt.%) being also (1.56 times) higher than the SFE yield (*ca.* 0.23 wt.% for SFE-3 and SFE-4), the stigmasterol Soxhlet concentration (as presented in Figure 14) is significantly lower than its supercritical counterpart, (25, 22 and 12% mg/(100mg$_{extract}$) for SFE-3, SFE-4 and Soxhlet, respectively).

Although SFE-3 and SFE-4 appear to have very similar supercritical extraction curves, when they are analyzed in terms of stigmasterol concentration in the extracts (Figure 14) that similarity weakens since SFE-3 exhibits a higher maximum concentration than SFE-4 counterpart (25 and 22% respectively).

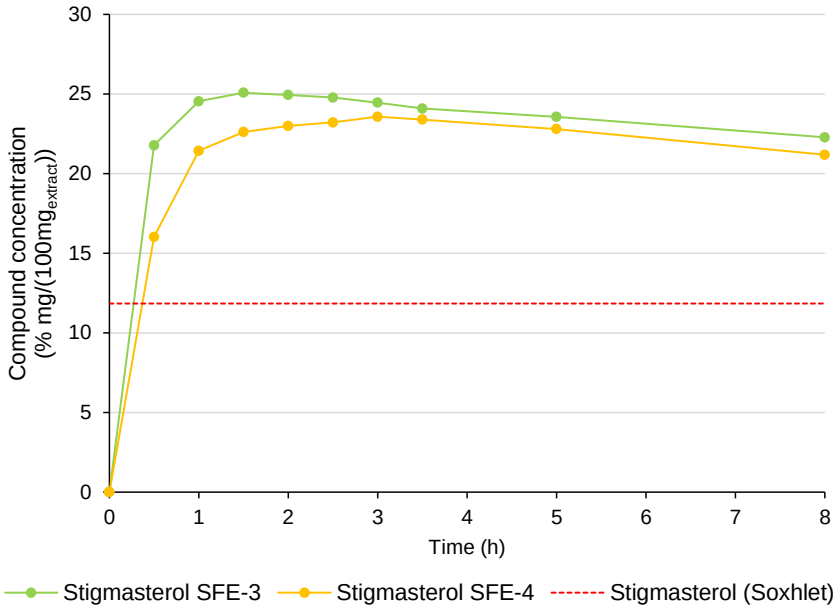


Figure 14 – Stigmasterol content of the extract along the time of SFE-3 and SFE-4 runs.

Additionally, special attention should be drawn to the stigmasterol concentration difference between the preliminary extractions (SFE-1, 31%; SFE-2 32% mg/(100mg$_{extract}$) in both cases a mixture of roots, stalks and leaves are included), and from the measurement of supercritical extraction curves (SFE-3, 25%; SFE-4, 22% mg/(100mg$_{extract}$); in both cases only stalks and leaves are included). Due to the lower Soxhlet extraction yield attained from roots (1.12 wt.%), and its lower stigmasterol concentration (see Table 7) (6.50% mg/(100mg$_{extract}$)) compared to the values attained from stalks (15.15% mg/(100mg$_{extract}$)) and leaves (13.45% mg/(100mg$_{extract}$)), it should be expected that SFE-3 and SFE-4 should attain higher stigmasterol concentration than SFE-1 and SFE-2. In the following the influence of the harvest season of *E. crassipes* is analyzed with the objective to justify this apparent inconsistency.

The variations of stigmasterol concentration between both harvested biomass extractions for Soxhlet (Table 11) and SFE (preliminary and OEC) can be imputed to various sources (Figure 15). One possibility is that although all biomass was randomly collected, different level of maturity between both harvested biomass could exist, reflecting this difference between stigmasterol concentrations. Another possibility could be related to the different harvest seasons, summer (1st Harvest) and winter (2nd Harvest). Portuguese winter, while mild, has significantly lower temperatures which affects the productivity and growth of a plant and also has less sunlight exposure than

the *E. crassipes's* native location. These fundamental aspects diminish photosynthesis capacity and therefore cellular growth.

Table 11 – Soxhlet stigmasterol concentration for both harvest seasons composed by roots and stalks.

Soxhlet extraction	% Stigmasterol mg/(100mg$_{extract}$)
1st Harvest	15.04
2nd Harvest	11.83

According to Hubert Schaller [38], sterols play an important metabolic role in cell proliferation and differentiation, where stigmasterol in association with cholesterol have an important role in cell growth and regulation of metabolic events. The graph in Figure 15 shows that the first harvest concentration of stigmasterol and cholesterol has higher values than the second harvest, thus supporting these reasoning.

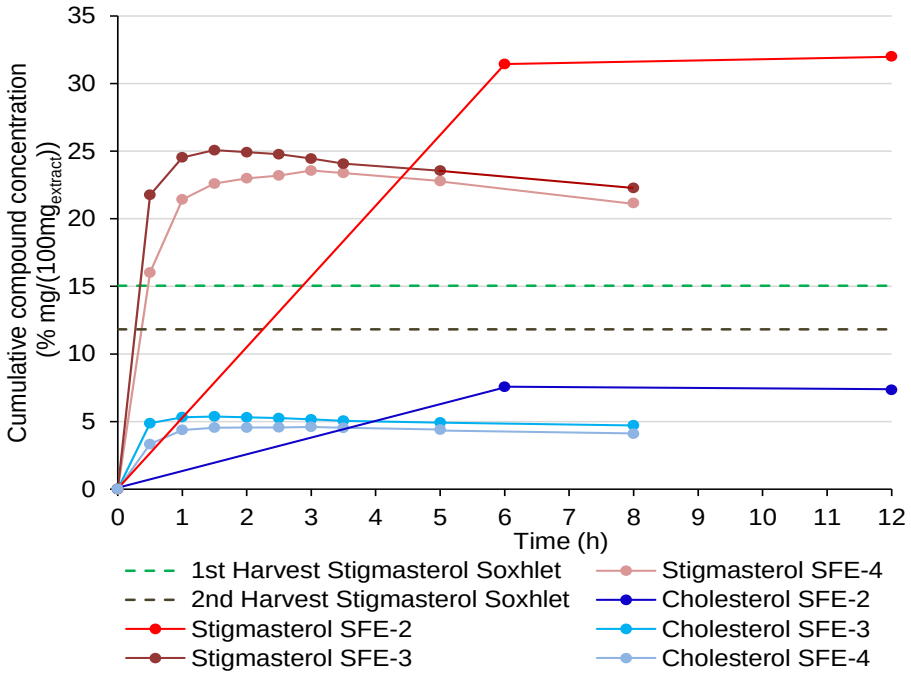


Figure 15 – Stigmasterol and cholesterol concentration of extracts obtained from both harvested seasons.

The stigmasterol concentration of each individual extract is represented in Figure 16 (left). This representation shows the precise Stigmasterol concentration of every collected extract over time. With this insight, we can state that SFE-3 exhibited a higher

individual concentration than SFE-4 (31 and 24% mg/(100mg$_{extract}$), respectively), along with higher fraction and stigmasterol yield at the first hour of extraction.

This type of analysis can be considered useful allowing for the appropriate selection of the most favorable extraction period, to maximize the concentration of a specific compound or family of compounds. Although this action would result in lower extraction yields, due to reducing the extraction time, it may become useful when dealing with costly purification processes, thus contributing for a better tradeoff between total/partial extraction yields, compound concentration and purification costs. However, if the extract concentration is not important within the industrial process, such tradeoff depends solely on the yield.

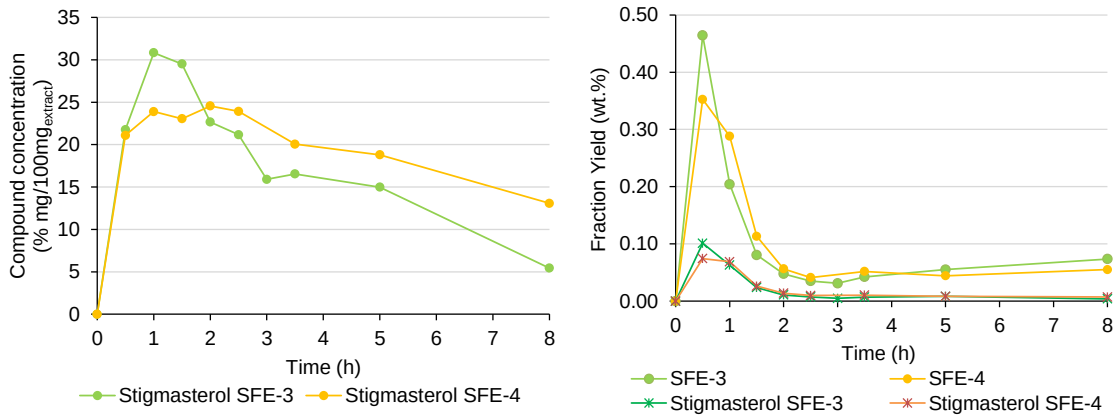


Figure 16 – Stigmasterol concentration (left) and total/stigmasterol yield (right) for the SFE-3 and SFE-4 runs.

Furthermore, while solute vapor pressure is only temperature-dependent, the pressure and temperature can be physically related in terms of density, viscosity and diffusivity. When variations of temperature and/or pressure are considered, these have major repercussions regarding fluid hydrodynamics, solubility and mass transfer, thus defining in great extent the performance of SFE systems.

Moreover, when at constant pressure and temperature suffers a variation, this action results in contrary effects upon solubility, since SC-CO_2 density (ρ) and vapor pressure of solutes exhibit conflicting behaviors (when temperature increases, ρ decreases and vapor pressure of solute increases). Upon examination of experimental data shown in Figure 13, Figure 14 and Figure 16 it becomes evident that although OECs present inconclusive data about temperature effect on the SFE, compound concentration/fraction and yield fraction presents relevant data about the influence of temperature on the extraction. While temperature increases from SFE-3 to SFE-4, its

negative effect due to density reduction prevails over the positive impact upon vapor pressure.

Additionally, it is possible to state that the optimal compromise between the extraction time and total/stigmasterol yield corresponds approximately to the first hour of extraction for the SFE-3 and SFE-4 runs (Figure 17) [86]. This optimum time value corresponds to the transition between the dominant transport mechanisms (external diffusion *vs.* intraparticle diffusion).

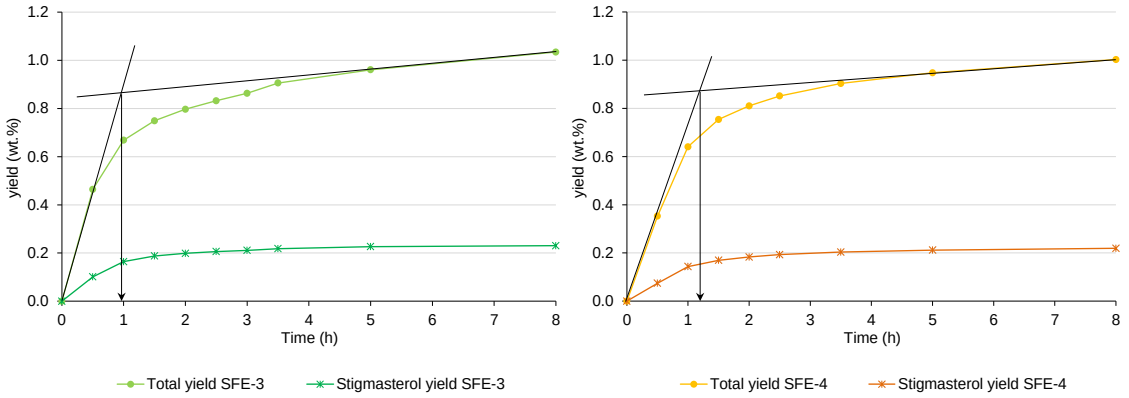


Figure 17 – Optimal extraction conditions for both SFE-3 (left) and SFE-4(right).

Within this time frame we can achieve a total extraction yield that corresponds to 65% of the total extraction for both SFE-3 and SFE-4 (yield values of 0.67 and 0.65% (wt.) respectively); a stigmasterol yield value that corresponds to 71 and 65% of the total stigmasterol obtained also for both SFE-3 and SFE-4 (stigmasterol yield values of 0.16 and 0.14% (wt.), respectively); and a stigmasterol concentration of 24.5 and 22.4% mg/(100mg$_{extract}$) also for both extractions.

Furthermore, and on the subject of the two overall extraction curves, it is possible to state that between SFE-3 and SFE-4, the former revealed the most appropriate extraction conditions for the recovery of stigmasterol, achieving higher concentrations and total/stigmasterol yield, while requiring lower temperature of operation.

3.2.3 MODELING

With the information provided from SFE-3, we now aim at the disclosure of the dominant mechanisms in this SFE experiment. As stated in section 1.4 the measured extraction curves were modeled with simple expressions based on Simple Single Plate (SSPM), Diffusion (DFM), Logistic (LM) and Desorption (DSM). The first two models focus on intraparticle diffusion while remaining two focus on interfacial mass transfer along the process. These models were applied to the extraction curves for both total and stigmasterol yields for the SFE-3 run. The fitted parameters and the respective statistical indicators are listed in Table 12, and Table 13 compiles the bed features required for DSM calculations.

Table 12 – Optimized parameters of the Diffusion, Simple Single Plate, Desorption and Logistic models for the SFE-3 run. The correlated responses are total extraction yield (TY) and Stigmasterol extraction yield (SY).

Logistic Model (LM)						
	b (h^{-1})	t_m (h)	R^2	AARD (%)	$b_{(SY)}/b_{(TY)}$	$t_{m(SY)}/t_{m(TY)}$
Total Yield	0.08	-219.6	0.826	31.8	3.94	0.28
Stigmasterol Yield	0.317	-62.3	0.882	22.1		
Desorption Model (DSM)						
	k_d (h^{-1})	R^2		AARD (%)	$k_{d(SY)}/k_{d(TY)}$	
Total Yield	0.124	0.847		29.1	4.6	
Stigmasterol Yield	0.569	0.954		13.8		
Simple Single Plate Model (SSPM)						
	D_m/δ^2	R^2		AARD (%)	$(D_m/\delta^2_{(SY)})/(D_m/\delta^2_{(TY)})$	
Total Yield	0.004	0.928		14.4	5.25	
Stigmasterol Yield	0.021	0.914		13.6		
Diffusion Model (DFM)						
	D_m/R_p^2	R^2		AARD (%)	$(D_m/R_p^2{}_{(SY)})/(D_m/R_p^2{}_{(TY)})$	
Total Yield	0.002	0.941		13.4	7.5	
Stigmasterol Yield	0.015	0.944		9.8		

Table 13 – Bed features necessary for Desorption Model (DSM) calculations.

ε	0.444
ρ_{solv} (kg m^{-3})	868.83
ρ_s (kg m^{-3})	208.32
H (m)	0.1
S (m^2)	0.004417

With regard to the determination factors obtained for the total extraction yield models, these were within 0.826 and 0.941, while the calculated average absolute relative deviations (AARDs) ranged between 13.4% and 31.8%. The plots of the modeled cumulative curves of total extraction yield are represented in Figure 18. It is worth noting

that LM and DSM provided the worse representation among all exhibited models. This difference between the interfacial mass transfer models (LM/DSM) and the intraparticle transfer models (DFM/SSPM) suggests that the dominant mas transfer mechanism in SFE-3 is the intraparticle diffusion. In fact, SSPM and DFM presented higher fitting adequacy with R^2 values of 0.928 and 0.941, and AARD values of 14.4% and 13.6%, respectively. This behavior was quite predictable since the cumulative curve in question possesses a small constant extraction rate period as discussed previously in section 3.2.2.

Analyzing the adjustable parameter (t_m) for the LM model, it showed negative values which possess no physical significance. Mezzomo *et al.* [60] and Domingues *et al.* [87] have also reported negative values when fitting LM to the SFE data. Such consistent finding revealed the inadequacy of the Logistic equation (originally developed as population growth model) to compute accurate interfacial mass transfer fluxes.

Regarding the poor results obtained by DSM, these evidenced that the concentration profiles are nonuniform inside the solid matrix and, as such, the mass transfer rate cannot be accurately associated to a single desorption step.

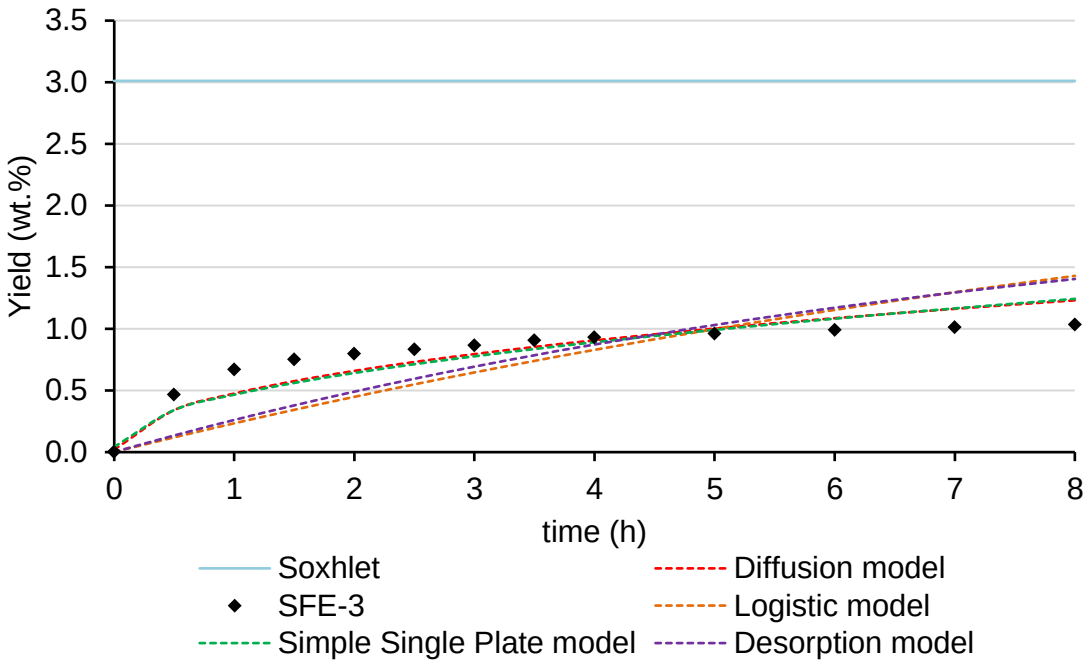


Figure 18 – Modeled cumulative curves for the total extraction yield of the SFE-3 run. Points are experimental data.

With respect to the stigmasterol extraction yield modeling (illustrated in Figure 19) LM and DSM also provided the worse representation of the experimental data. As discussed within the analysis of the total extraction yield models, both interfacial mass transfer equations (LM and DSM) presented the worst fitting results with R^2 values of

0.882 and 0.954, and AARD values of 22.1% and 13.8%, respectively. Between both intraparticle diffusion models (SSPM and DFM), DFM presented the higher fitting adequacy with an AARD value of 9.8%, which indicates that besides intraparticle diffusion being dominant, the process resembles more of a spherical geometry than a planar one. Furthermore, regarding the best fitting model, the DFM, the adjustable parameter (D_m/R_p^2) presented values of 0.002 and 0.015 for the total and stigmasterol yield curves. Because R_p^2 is biomass dependent, thus, constant for the SFE-3 run, the variation between the adjustable parameters for both models are only dependent of the solute diffusivity in the solid (D_m). This parameter ratio between stigmasterol yield model and the total yield one presented in the right column of Table 12, translated in a higher solute diffusivity (7.5 times higher) of stigmasterol than an average solute diffusivity. This higher solute diffusivity of stigmasterol molecules within the solid matrix translates, as already discussed in section 3.2.2, as a higher selectivity of SFE-3 run for this particular molecule.

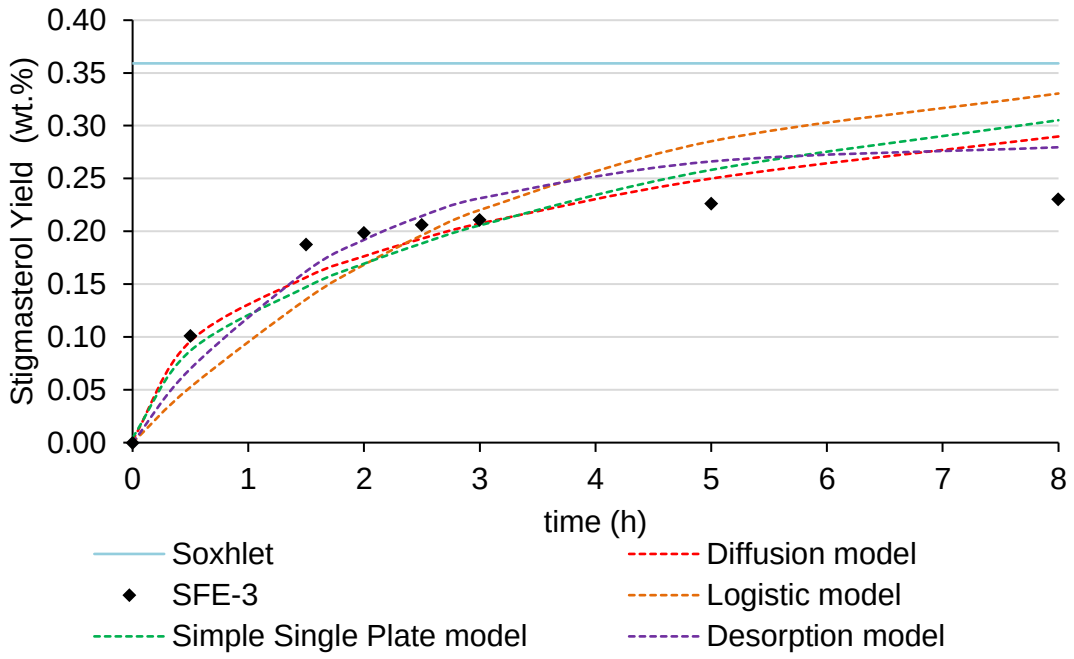


Figure 19 – Modeling cumulative curves of stigmasterol extraction yield for the SFE-3.

4. CONCLUSIONS AND FUTURE WORK

Conclusions

This thesis aimed to study the *Echhornia crassipes's* extractives in order to present a freshly new insight on this invasive species which is considered the fastest growing plant in the world.

This essay presents detailed information concerning the chemical characterization of the lipophilic fraction for each morphological part of the plant, where sterols were the most abundant family within the lipophilic extractives. Among the identified sterols special attention should be drawn to the concentration values attained by stigmasterol, namely in stalks (15.15% mg/(100$mg_{extract}$)) and leaves (13.45% mg/(100$mg_{extract}$)). The characterization of the hydrophilic fraction of *E. crassipes* revealed that leaves are the morphological part of this species that possess both higher antioxidant activity and higher total phenolic content.

Regarding the supercritical fluid extractions, and although the total yields were lower than that attained by conventional methods (Soxhlet), this greener technique almost doubled the concentration of stigmasterol (25% mg/(100$mg_{extract}$)) of its Soxhlet counterpart (13% mg/(100$mg_{extract}$)), thus being more selective for this particular molecule. Furthermore, the use of cosolvent in the SFE contributed negatively to the stigmasterol concentration, due to the increased solubilization of other competing molecules.

Additionally, temperature variation between SFE runs had little impact on total and stigmasterol yields. In relation to the stigmasterol concentration in the extract, the influence of temperature was consequently small, though *ca.* 2-5% variation could be observed during the first hour of extraction. Furthermore, and on the subject of the two overall extraction curves (OEC), it is possible to state that between SFE-3 and SFE-4 runs, the former revealed the most appropriate extraction conditions for the recovery of stigmasterol, *i.e.* 40ºC, 200bar and a constant CO_2 mass flow rate of 11 g min^{-1}.

Moreover, it is possible to indicate that for the SFE-3 conditions, the optimal compromise between the extraction time and total/stigmasterol yields corresponds to the first hour of extraction. Within this time interval we achieve 65% of the total extraction for SFE-3 (yield value of 0.67% (wt.)); a stigmasterol yield value that corresponds to 71% of the total stigmasterol obtained for SFE-3 (stigmasterol yield value of 0.16% (wt.)); and a stigmasterol concentration of 24.5% mg/(100$mg_{extract}$).

With respect to the applied models we aimed at the disclosure of the dominant mechanisms in the SFE-3 experiment. Between them, the Logistic and the Desorption models (LM/DSM) achieved the worst representation for both total and stigmasterol yield, while the Diffusional and the Simple Single Plate models (DFM/SSPM) presented better fittings. This difference between interfacial models (LM/DSM) and the intraparticle diffusion ones (DFM/SSPM) suggest that the dominant mass transfer mechanism in the SFE-3 run is the internal diffusion. In addition, by confronting the adjustable parameters for the total yield and the stigmasterol yield within the applied models, one can restate that the supercritical fluid extraction is highly selective to the stigmasterol molecule, presenting a higher solute diffusivity.

Future work proposal

For future reference, further analysis should be conducted on this species extractives, specifically its high yield hydrophilic fraction. Different solvents and methods should be applied in order to extend the composition knowledge of the plant along with the prospect of finding new compounds with tumor inhibition, antimicrobial, anti-inflammatory, antifungal and antibacterial properties [19–27]. These extracts should be analyzed by high performance liquid chromatography (HPLC) and screened for their possible bioactivity and medicinal applications among others.

Within this thesis, lipophilic and hydrophilic extractives which represented approximately 10 to 30% (wt.) of total biomass were studied. Following the much applied biorefinery concept, it would be of utmost importance to study the chemical composition of the remaining biomass, and consequently, its possible valorization.

Regarding the biomass and its harvest, further study should be performed concerning the oscillations of chemical composition and concentration with different seasons. Within this thesis, stigmasterol concentration varied from the first harvest to the second one, suggesting that the genesis of this fluctuation could perhaps be linked to the different harvest seasons (summer/winter), different levels of plant maturity, or even, to some sort of undetected illness. Bearing these aspects in mind, further studies should be accomplished regarding the biomass in order to maximize the extraction yields and ultimately its stigmasterol concentration.

As for the supercritical fluid extraction, an optimization of the operating conditions should be considered in order to achieve optimal total and stigmasterol extraction yield

along with its extracts concentration. In pursuance of this optimization, a design of experiments (DOE) should be taken into consideration.

Furthermore, according to recent publications regarding the use of a focused ultrasound extraction (FUSE) in order to achieve higher extraction yields [88], this tool could help achieve higher yields by improving mass transfer rate.

If future studies of hydrophilic extracts from *E. crassipes* reveal to be a source of added value compounds, SFE studies should be also performed. Moreover, and bearing in mind the presented work in this thesis, sequential extractions could/should be taken into consideration. After optimization of stigmasterol extraction is performed, which would lead to an impoverishment of lipophilic matter within the biomass, sequential SFE with the use of cosolvent could be accomplished in order to retrieve such molecules of added value with a possible higher selectivity.

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